ASPECTS ON THE FORMATION AND TEMPERATURE MODIFICATION OF LEAF SURFACE WAX IN *Brassica napus* L.

by

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, it contains no material previously published or the result of work by another person, except where due reference is made in the text.

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Canberra,
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SUMMARY

Electron microscopy, gas-liquid chromatography and mass spectrometry have been used to elucidate many of the phenomena concerned in the formation and temperature modification of leaf surface waxes in *Brassica napus* L.

A characteristic wax pattern was observed for plants growing over a range of temperature regimes. The change observed in wax pattern was one of wax rods at low growing temperature and complex wax plates at high growing temperature (up to 36 °C).

Low and high temperature waxes were found to differ chemically by a small but consistent reduction in the major C_{29} and C_{31} hydrocarbons accompanied by an increase in the C_{29} symmetric ketone. The presence of C_{42} to C_{45} ester compounds in *Brassica* spp. leaf wax is reported here for the first time.

The effects of different growing temperatures, wax mutants and dewaxing herbicides did not reveal any marked cytological differences in epidermal cell fine structure, which could be related to wax synthesis.

An autoradiographic study implicated endoplasmic reticulum and Golgi vesicles in the manufacture and transport of waxes or wax precursors respectively. Movement of the waxes to the leaf surface appears to take place purely by diffusion across the cell wall and the adjacent subcuticular region whereafter wax reaches the surface by means of cuticular channels and surface pores.

The cuticular pores were found to play no role in the formation of wax patterns characteristic for a given growing temperature. This pattern indeed was largely attributed to the small differences in wax chemistry and conditions under which the wax crystallised.

The 'growth ring' appearance characteristic of wax rods was shown not to result from extrusion of the wax on the leaf surface as has been suggested previously.

Low temperature leaf wax was found to be modified significantly within hours of transferring the plant concerned to a higher temperature stress. Such transformations were quite phenomenal and were found to be quite irreversible.

CONTENTS

GENERAL INTRODUCTION

1. THE LEAF CUTICLE — ITS NATURE AND FUNCTION

(i) The Problem of Definition

Aerial parts of the primary plant body are invested with a protective cuticle. Consisting primarily of lipoidal substances, the cuticle overlies and merges into the outer walls of the epidermal cells. Because of this close association with the epidermis, a strict morphological definition of the cuticle has proved difficult. Indeed, the term has been used in different senses and modified in the literature (Anderson, 1935; Priestley, 1943; van Overbeek, 1956; Goodman, 1962; Sitte & Rennier, 1963; Crisp, 1965; Martin & Juniper, 1970).

Crisp (1965), following definitions of Esau (1953), defined the outer epidermal wall as comprising stratified layers of cellulosic, pectinaceous and lipoidal materials, the outermost layer being the cuticle. This definition would suffice in most cases, except that the three layers are by no means always clearly defined. The pectic layer may be virtually absent, with the cellulosic layer and the cuticle merging gradually into one another as in *Clivia* (Roelofsen, 1952). In such circumstances, an arbitrary distinction is usually made between the cuticularised (or cutinised) cell wall layer containing both cellulose and cuticular substances, and the cuticle proper which contains no cellulose. The cuticle is thus defined in a positional sense.

(ii) Morphological Description

The recent review by Martin & Juniper (1970) sets out in detail the diversity of cuticular morphology as well as the basic similarities common to all. If one regards the pectic layer simply as a boundary, the cuticle lying external to it conforms to the common biological pattern of a reinforced matrix. The three-dimensional polyester meshwork contains within its interstices varying proportions of lipid substances, denoted usually as waxes, though with no precise chemical sense intended. Overlying this so-called 'cutin' layer is the layer of 'epicuticular waxes'. Though this name implies that waxes lie on the cuticle, and are thus distinct from it, it is considered more appropriate, structurally and morphogenetically, to regard the waxy layer as the superficial part of

the cuticle. For one thing there is every likelihood that the surface waxes and the embedded waxes are continuous, if only by means of surface pores (Hall & Donaldson, 1962; Hall, 1967a). Schieferstein & Loomis (1959) reported that only about 50% of the species they studied had waxy leaf cuticles. It has been shown since (D.M. Hall, personal communication) that the former authors, because of the replica methods used for their observations, failed to recognise the large flat sheets of non-structural wax on their 'non-waxy' species. It is still true to say, however, that the proportions of cutin to wax (embedded and superficial) vary widely between species.

(iii) Basic Chemistry

Chemically, the cutin framework is a polyester consisting of polymerised dicarboxylic and hydroxy-substituted aliphatic acids. The hydroxy acids are commonly substituted in the omega-position allowing head to tail condensation while mid-chain substitution, frequently in the 9,10-position, permits three-dimensional polymerisation. The result is a closely-meshed elastomer with a tendency to lamination (Linskens *et al.*, 1965; Eglinton & Hamilton, 1967; Martin & Juniper, 1970).

The chemistry of plant waxes has most recently been reviewed by Eglinton & Hamilton (1967), Mazliak (1968), and Martin & Juniper (1970), while rapid advances in their characterisation has been discussed by Lindeman & Annis (1960), Eglinton *et al.* (1966), and Holloway & Challen (1966). In general, plant waxes are mixtures of long chain alkanes, primary and secondary alcohols, ketones, ketols, acetals, esters and acids plus true waxes in the chemical sense. At first sight, these compounds may appear simple; however the variability and complexity of plant waxes cannot be over-emphasised.

(iv) Physiological Functioning

The cuticle and its associated waxy layer has been ascribed numerous barrier functions between the environment and the plant body.

(a) Suppression and stimulation at the leaf surface of infection by fungi has received a considerable amount of attention. The results, however, have been rather conflicting, and reference is made to Martin & Juniper (1970), where a detailed account of the work in this field is given. What appears to be of major importance in the role of the surface wax as a defence against pathogenic microorganisms, is its hydrophobic nature which makes it difficult for organisms to establish themselves.

(b) While a high degree of glaucousness does not necessarily indicate extreme waxiness, the presence of surface wax has an effect on the light reflection characteristics of leaves. Variations in light reflectance due to leaf surface features have been studied by Billings & Morris (1951), Moss & Loomis (1952), Cameron (1964), Howard (1966), and Armstrong (unpublished data). The light scattering character of the rough textured wax layer and the light absorbing powers of polyphenolics in the cutin may well shield the plant from excess ultraviolet radiation.

(c) Of agricultural importance is the contact angle made by water drops on a leaf surface. A considerable amount of work has been done in measuring and correlating the magnitude of the contact angle with surface roughness and waxiness. The more recent workers in this field include Thrower *et al.* (1965), Wortman (1965), Martin (1966), Sargent (1966), Troughton & Hall (1967), and Armstrong (unpublished data). Holloway (1969) has studied the orientation of constituent molecules in surface wax in relation to water repellency but found that hydrophobic properties generally give little indication of chemical composition. Penetration of externally applied solutions, particularly in the study of herbicides, has been investigated more recently by Franke (1964, 1967), Hallam (1964), Middleton & Sanderson (1964), Martin (1966), Sargent (1966), and Sands (1972).

(d) The more recent work on cuticular transpiration indicates that surface waxes are important in preserving the water balance of the plant. Increased transpiration due to disruption of the wax surface by mechanical or chemical means has been noted by Pfeiffer *et al.* (1957), Hall & Jones (1961), Daly (1964), Horrocks (1964), Bain & McBean (1967), Possingham *et al.* (1967), and Denna (1970). Grncarevic & Radler (1967) have noted that aliphatic components such as the hydrocarbon, alcohol and aldehyde fractions of the surface wax gave the greatest reduction in transpiration from grape cuticle. Cuticular transpiration with surface wax was found to be less than 10% of that for a dewaxed grapeskin. Some workers have suggested that the cuticle and its associated waxiness may have significant permeability to carbon dioxide (Dorokhov, 1963; Jeffree *et al.*, 1971).

2. BIOSYNTHESIS OF CUTICULAR WAXES

(i) Genetics of Wax Inheritance

The chemical genetics of wax formation have recently been examined for *Pisum* and *Brassica* spp. by Macey & Barber (1969, 1970a,b). In *Brassica* mutants, the genes of at least two different loci were found to be responsible for the glossy leaf character of the mutant concerned. In both studies, the mutants contained reduced amounts of paraffin, ketone and secondary alcohol fractions, while the aldehyde primary alcohol and ester components were more prominent when compared with the normal waxy plant. The genetic control of diketone fractions, in relation to the synthesis of wheat and barley waxes respectively, has been examined by Barber & Netting (1968) and Wettstein-Knowles (1972). Clines in glaucousness have been correlated with changes in frost severity. Such clines appear to represent changes in allelic frequency at one or more loci controlling the development of wax (Barber, 1955; Barber & Jackson, 1957). Macey (1967) reported that it is likely that several genes are active in determining different surface wax patterns.

(ii) Variations in Wax Amount, Type and Chemistry

Variations in cuticular composition within species have been demonstrated, and have been shown to depend on leaf age, leaf surface (adaxial or abaxial), and environmental growth conditions (Kurtz, 1950; Mueller *et al.*, 1954; Skoss, 1955; Juniper, 1959b, 1960; Baker, 1963; Hall & Donaldson, 1963; Leigh and Matthews, 1963; Whitecross, 1963; Baker & Martin, 1967; Eglinton & Hamilton, 1967; Herbin & Robins, 1968a,b, 1969; Hallam & Chambers, 1970; Hawthorn & Stewart, 1970; Banks & Whitecross, 1971; and Whitecross & Armstrong, 1972).

The more recent work on the biosynthesis of plant surface wax components has made use of ^{14}C-labelled fatty acid precursors in an attempt to evaluate the mechanisms involved (Kaneda, 1966, 1967, 1968; Kasprzyk & Wojciechowski, 1969; Kolattukudy, 1965, 1966, 1967a,b,c, 1968, 1970a,b,c; Kolattukudy *et al.*, 1968; Kolattukudy & Tsui-Yun, 1970; Kolattukudy & Walton, 1972; Marekov *et al.*, 1968; and Mitchie & Reid, 1968). A detailed account of the various biosynthetic mechanisms in relation to leaf waxes has been recently reviewed by Kolattukudy (1970b,c). Alkanes are considered to be formed from fatty acids either by elongation followed by decarboxylation or by 'head to head' condensation between two biochemically dissimilar fatty acids followed by

specific decarboxylation of one of them. Fatty acyl-CoA is reduced to the aldehyde which in turn is reduced to the alcohol. The alcohol is then esterified with acyl moieties from acyl-CoA or phospholipids.

(iii) Biosynthetic Site and Pathway for Wax Precursors

While it is generally agreed that the epidermal cell layer of plant leaves is the site for the production of leaf wax precursors (Kolattukudy, 1968, 1970b,c), there is much speculation within the literature as to how these components traverse the cell wall and cuticle to become established on the leaf surface (Linskens *et al.*, 1965). Whether the mechanisms involved are achieved via some specific wax microchannel cum surface pore arrangement, or else by purely diffusive means along a concentration gradient, or perhaps by a combination of these factors, has not been clearly established for any plant. Even assuming that the mechanisms involved could be determined for a particular plant species, one could not be certain that the same pathway operated in all waxy plants, though differences between species would most likely be small.

3. AIMS OF THIS INVESTIGATION

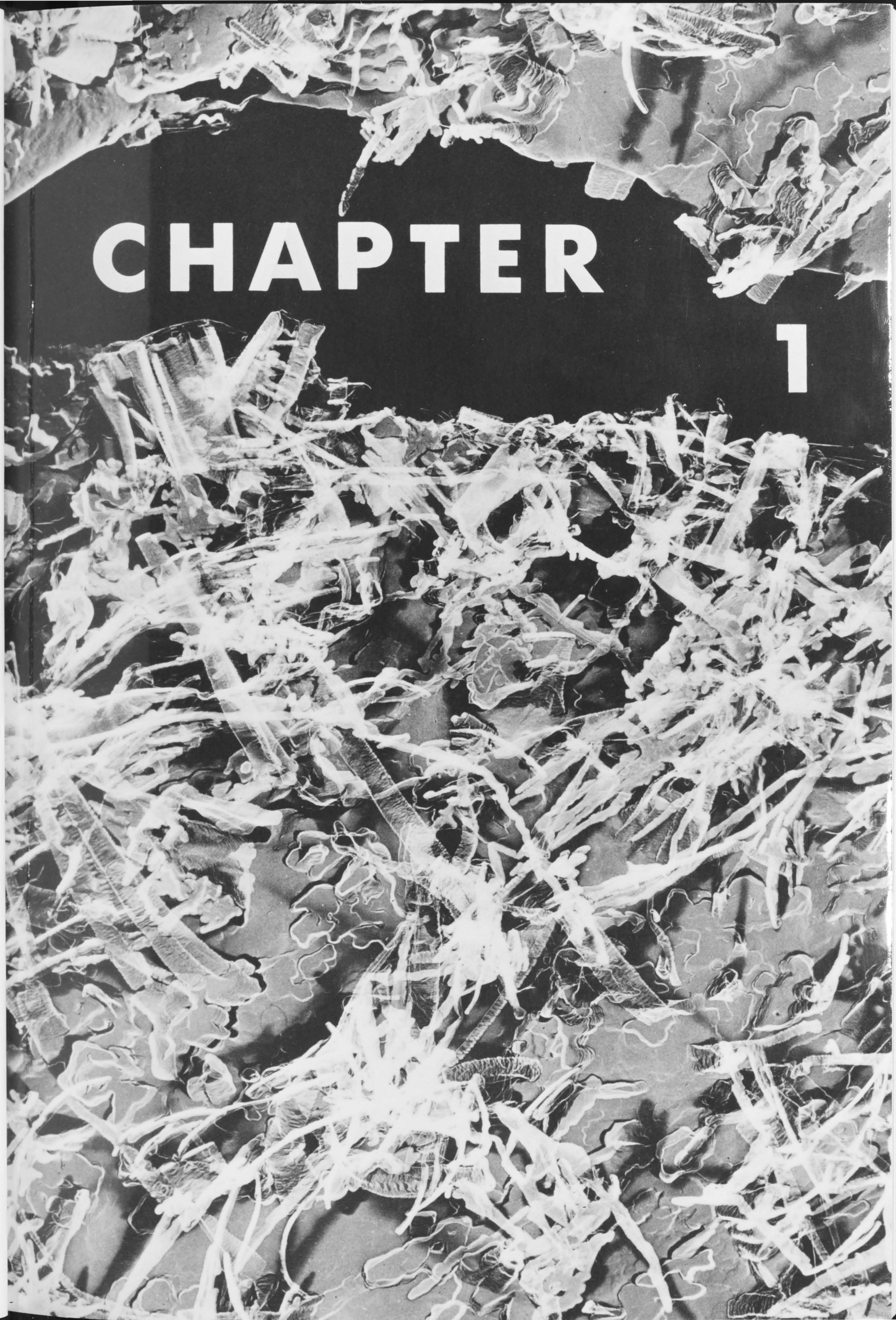
In view of the significant qualitative differences observed in the leaf surface wax pattern of *Brassica napus* when grown under a variety of temperature regimes (Whitecross & Armstrong, 1972), it seemed appropriate to make a comprehensive study of this plant in the hope of answering some specific questions on formation of leaf surface wax and its modification in response to temperature changes, viz.

(i) What specific organelles within the cells of a leaf are responsible for the manufacture of wax precursors?

(ii) What pathway operates in the transport of waxes or their precursors from the cells across the outer cell wall and cuticle regions, and how do the waxes finally reach the leaf surface?

(iii) Once on the leaf surface, is the wax pattern characteristic of a particular growing temperature determined by morphological and surface characteristics of the leaf, the chemical composition of the wax, or simply the physical conditions encountered by the wax as it deposits?

(iv) How readily adaptable is a growing plant to alter its leaf surface wax pattern in response to a rapidly altered temperature regime?



CHAPTER 1

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MODIFICATION IN FINE STRUCTURE OF EPIDERMAL CELLS AND ASSOCIATED SURFACE WAXES

INTRODUCTION

Waxy substances are components of the leaf cuticle from the very earliest stages of leaf development. During leaf expansion, wax is found on the surface in a semi-crystalline form and appears to be produced continuously during this phase of growth. Regeneration of structural wax on damaged areas of the leaf surface may continue until a late stage of leaf expansion (Juniper, 1960; Hallam, 1970c).

The presence of structural wax on plant surfaces raises the question of how such fine structures develop and whether the modification attributed to the changing of environmental factors can be characterised.

Whitecross (1963) has described the effects of light, temperature and water stress on the structure and composition of leaf wax of *Brassica oleracea*. More recently the effect of light and temperature on the waxes of *Brassica napus* has also been examined (Whitecross & Armstrong, 1972). In each case, structural alteration of the wax resulted from different growing temperatures, while the effect of light was to alter the wax quantitatively. Water stress apparently affected the chemical proportions in the wax (Whitecross, 1963).

The earliest investigations on the effects of varying growth temperatures have been restricted in several ways. For instance, the range did not include the region of possible stress above, say, 30 °C. Further, the observed variation in fine structure of surface wax represented only the end result of a chain of physiological events capable of being affected by temperature. There was thus a definite need to extend the investigation to include a study of the effects of higher temperatures and also to examine the effects throughout the range at the site of wax synthesis in the cytoplasm of the epidermal cells themselves (Kolattukudy, 1968, 1970b).

Also in need of further investigation were the non-waxy *Brassica* mutants known to produce some surface wax of a non-structural form

whereby the leaves appear green as opposed to those of the typically glaucous waxy varieties. Another greening effect has been observed as a result of inhibiting wax synthesis with T.C.A. (Kolattukudy, 1965, 1970b,c; Hallam & Juniper, 1971).

Pre-emergent soil treatment with T.C.A. almost completely suppressed surface wax formation in peas (Juniper, 1959a; Dewey *et al.*, 1961) while changes induced by related herbicides on epidermal cell membrane systems have been observed by Hallam (1970a).

The question arose as to whether the natural mutants, in which wax formation is affected, have anything in common at the cellular level with plants affected by T.C.A. (trichloroacetic acid), or other related compounds such as dalapon (2,2-dichloropropionic acid). This investigation sought to make this comparison possible also.

Finally, the pathway linking the site of wax formation with its ultimate repository demanded examination. The effects of temperature, such as those observed, may have resulted neither from direct effects at the cuticular surface nor from effects at the metabolic level in the cytoplasm, but rather from effects on the membranes, cell walls and cuticle which the wax or its precursors traverse.

METHODS

Light Microscopy

Light micrographs of leaf cross sections were prepared by cutting 1 μm sections of epoxy-embedded material and staining with a solution of 1% aqueous Azure B and methylene blue containing 1% borax (A.E. Ashford, personal communication).

Low power leaf surface characteristics were prepared by the use of replicating tape (Ladd Corporation).

Scanning Electron Microscopy

Fresh representative leaves were cut and glued onto specimen stubs. The exposed leaf surface was initially coated with carbon (~ 60 nm) followed with 60/40 gold/palladium alloy. The specimens were viewed directly in a JEOL scanning electron microscope and photographed on to 4" × 5" Kodak EKTAPAN film.

Transmission Electron Microscopy

Thin Sections

Leaf material was prepared according to the following schedule:

1. Leaf pieces < 1 mm^2 were rapidly cut from a representative leaf and placed in 3% glutaraldehyde in 0.025 M sodium phosphate buffer, pH 7.2, for two hours at room temperature, vacuum infiltration being applied where necessary.

2. The tissue was washed in 0.025 M sodium phosphate buffer, pH 7.2, for a minimum of forty-five minutes, generally one hour with four changes.

3. Post-fixation was carried out in 2% osmium tetroxide in the same buffer for one and a half hours at room temperature.

4. The tissue was further washed in the same buffer for ten minutes (two changes) before being dehydrated in a graded series of acetone solutions (20% — 30 minutes; 40% — 25 minutes; 60% — 20 minutes; 80% — 15 minutes; and 100% (two changes) — 15 minutes, at room temperature).

5. After dehydration the tissue was infiltrated in 1:1 acetone-Texas mixture for thirty minutes followed by a one hour infiltration (two changes) in 100% Texas mixture at room temperature. Vacuum infiltration of the embedding medium was not used. (Texas I mixture — Araldite M, Epon 812, D.D.S.A., dibutyl phthalate, DMP-30 (Mollenhauer, 1964).)

6. Polymerisation was carried out in silicone rubber moulds at 60 °C for six hours followed by four hours at 85 °C.

7. Sections were cut with a diamond knife and supported on 200- or 300-mesh uncoated copper grids. Staining was carried out in uranyl acetate (saturated 50/50 aqueous ethanol) and Reynolds lead citrate (Reynolds, 1963).

8. Sections were examined in a Philips 200 electron microscope at 80 KV and micrographs recorded on Kodak Estar base electron microscope film.

9. Good preservation of leaf surface waxes in thin section was attained by this schedule. Visualisation of the waxes in electron micrographs was intensified where necessary by an increase in the lead staining time.

Leaf Surface Replication

The carbon replica technique used in these and previous studies was a simplified version of that originally used by Juniper & Bradley (1958).

The simplification resulted mainly from using only one plastic backing layer prior to stripping from the leaf, and it produced consistently satisfactory results in an appreciably shorter time (Whitecross & Armstrong, 1972).

1. A leaf was cut into pieces approximately 1.5 mm long and 0.5 mm wide.

2. A glass microscope slide was coated with Bedacryl 122X stock solution (ICIANZ Ltd).

3. The leaf pieces were carefully transferred onto the tacky Bedacryl layer which set relatively quickly thus minimising leaf shrinkage.

4. The leaf pieces were then subjected to a vacuum $\sim 3 \times 10^{-5}$ mm Hg in an Edwards 12E6 vacuum coating unit and shadowed with Pt/C at 40°. A tight coil of 0.1 mm diameter Pt wire at the point of contact between two lathe-turned carbon rods, one as a cylindrical peg and the other steeply conical comprised the Pt/C evaporation assembly.

5. Carbon alone was then evaporated at 8×10^{-4} mm Hg in a series of brief 20V/30A bursts. A steeply conical carbon rod against a bevelled carbon rod comprised the carbon evaporation assembly.

6. The leaf pieces were immediately flooded with 15% Bedacryl in benzene and drained by tilting the slide.

7. When the backing layer of plastic was thoroughly dry (24 hours), a sharp razor blade was used to score the plastic and carbon films into convenient grid-sized pieces.

8. The combined plastic-carbon film was stripped from the leaf surface with fine forceps by lifting up one corner and carefully pulling it back.

9. The sections of plastic-carbon film were first washed in acetone to remove the plastic, and finally in 50/50 acetone/chloroform to remove any last traces of plastic or adhering leaf wax, before being picked up on a grid and dried.

10. Replicas were examined in the electron microscope and photographed onto Agfa Scientia 23D56 plates. The positive plates so obtained were reversed by a 1:1 contact printing onto Ilford N5.31 film, or to Kodak Commercial Ortho film.

Plant Growth

In order that the aims of the various sections of the work might be carried out and to allow the possibility of correlating results, growth

and sampling methods were standardised.

The plants used exclusively in this work were *Brassica napus*, variety Dwarf Essex (rape) representing the normal waxy plant and a non-waxy (gl_3) mutant, *Brassica oleracea*, variety acephala (Thompson, 1963; Macey & Barber, 1970b).

All plants were grown under the controlled environmental conditions available at the CERES Phytotron, C.S.I.R.O., Canberra. Seeds were fumigated with methyl bromide prior to germination in 24/19 °C. Single seedlings were transplanted into 5" pots containing a 60/40 perlite/ vermiculite mixture. All seedlings were established for ten days before being transferred to their respective temperature treatments. Day/night temperature regimes of 15/10 °C, 18/13 °C, 21/16 °C, 24/19 °C, 27/22 °C, 30/25 °C, 33/28 °C and 36/31 °C were available for plant growth. Plants were maintained under a sixteen hour photoperiod regime, natural light being supplemented by artificial incandescent lighting. Relative humidity was always greater than 40%.

All plants were given applications of nutrient solution in the morning and water in the evening. To avoid any water stress, the high temperature treatments were given an additional watering at midday (27/22 °C) or alternatively left standing in 1" of water (30/25 °C, 33/28 °C and 36/31 °C). The non-waxy (gl_3) mutant was grown only under a 24/19 °C regime.

Extreme care was taken at all stages of plant growth to avoid water and mechanical contact with the leaf surfaces.

Herbicide Treatment of Plants

In the application of herbicides, all treatments were applied to the root system only, plants being maintained in their normal perlite/ vermiculite mixture. Two-week old test plants, kept under a 24/19 °C temperature regime were treated daily with 1.5×10^{-4} moles of T.C.A. or alternatively with 5×10^{-5} moles of dalapon as the sodium salt. No attempt was made to add quantitative amounts of herbicide to the plant system or specific leaves. Initial investigations showed that the amounts added produced a striking reduction of leaf wax content without having caused obvious morphological changes in the plants. In *Brassica*, $1\text{-}2 \times 10^{-5}$ M T.C.A. in a single leaf inhibited surface wax synthesis by 50% (Kolattukudy, 1965).

Treatments were continued for ten days with the plants subsequently maintained under a normal watering regime for a further five days prior to sampling. This procedure was adopted to avoid any direct herbicide application effect.

Sampling

Leaf sampling, unless otherwise stated in the text, was carried out after the plants had been in their respective treatments for four weeks. The plants were chosen at random from a batch of treatment plants.

Disregarding the small apical leaves, the fourth visible leaf from the apex was taken as a representative sample for each plant unless otherwise stated. This leaf was almost fully expanded showing no signs of senescence. The principle involved in this sampling method was to compare leaves at a similar physiological age from the point of view of cuticular development. Variation in the development of leaves due to the effect of different treatments made the sampling of a particular node number from the cotyledons an unsatisfactory method of representative sampling.

In small-scale sampling of isolated leaves, a representative area of lamina from each was selected avoiding mid-rib, major veins and leaf margins.

RESULTS

(i) Effect of Growing Temperature on Epidermal Cells

Plate 1.1 a,b & c represents light micrographs of cross sectioned leaf tissue while plate 1.2 a,b & c illustrates surface replication views of leaves grown at 15/10 °C, 24/19 °C and 36/31 °C respectively. Average leaf thickness for the three respective temperature conditions were 0.28, 0.24 and 0.11 mm (method of measuring leaf thickness is detailed in Appendix I).

Throughout the fine structure study of *Brassica* epidermal cells, the tonoplast was frequently seen to protrude into the vacuole and was often constricted so as to form apparently isolated spheres of membrane within the vacuole. Degenerated organelles frequently appeared in the centre of these membrane spheres. Good preservation of other cytoplasmic structures would appear to rule out fixation artifacts as an explanation of these structures.

While this study was essentially concerned with the investigation of the upper epidermal layer, preliminary work showed the lower layer to be similar in all respects of fine structure except for actual cell size. No detectable fine structure differences were found between small developing leaves and fully expanded leaves for any particular temperature treatment. Only the cell wall thickness was observed to increase slightly in the more fully expanded leaves.

Plate 1.3 a,b & c illustrates the fine structure detail of the outer epidermal cell wall and adjacent cytoplasm for rape grown under a 15/10 °C temperature regime. Although the cytoplasmic layer for this treatment was relatively thin, at times little more than the thickness of one peripheral strand of endoplasmic reticulum, considerable activity must occur since appreciable amounts of wax are produced under these conditions (Whitecross & Armstrong, 1972).

Mitochondria and Golgi bodies are present in the cytoplasm while ribosomes, endoplasmic reticulum and vesicles make up the bulk of the cytoplasmic components. Large vesicles are frequently found associated with Golgi bodies. The plasmolemma was observed to be highly irregular in outline, frequently extending pseudopodia-like protruberances into a hyaline area corresponding to the inner cell wall.

The microfibrils making up the cell wall lamellae lie parallel to the leaf surface. These cell walls generally exhibit a staining gradient from the plasmolemma to the cuticle. No evidence was seen of intrusion of cytoplasmic vesicles into the cell wall despite the irregular plasmolemma mentioned earlier nor was any microchannel structure extending from the plasmolemma across the cell well to the cuticle ever observed.

The cuticle was observed to be consistently irregular in appearance although its overall thickness was relatively constant. Epicuticular wax structures are frequently present, their outline resembling that observed in replication studies (Whitecross & Armstrong, 1972). Even the consecutive ring type structure of wax rods has been confirmed in thin section studies. Preservation of surface waxes in thin section has been achieved without special fixation techniques or wax stabilisation procedures (Hallam, 1964, 1970b).

Plate 1.4 a,b & c illustrates the epidermal cell fine structure for rape grown under a 24/19 °C temperature regime. Structurally the

cytoplasm was similar to that observed for the 15/10 °C treatment although there was a general increase in the thickness of this layer and a proportionate increase in the number of cell organelles present. Conversely, there was a consistent decrease in the thickness of the cell wall. The cuticle, however, was similar both structurally and dimensionally to that observed in the 15/10 °C treatment.

Plate 1.5 a,b & c illustrates the epidermal cell fine structure for rape grown under a 27/22 °C temperature regime. The fine structure of the cytoplasm was very similar to that observed in the previous treatment. There was an apparent increase in ribosome number and a decrease in the Golgi frequency. There was a continuing decrease in cell wall thickness while the cuticle structure was consistent with that previously observed.

Plate 1.6 a,b & c illustrates the epidermal cell fine structure for rape grown under a 36/31 °C temperature regime. In contrast to the three lower temperature treatments, the cytoplasmic organelles, tonoplast and plasmolemma showed definite signs of degeneration. A decrease in the amount of cytoplasm was observed with a consequent decrease in endoplasmic reticulum, ribosomes and cytoplasmic vesicles. Irregularities in the tonoplast and plasmolemma were not nearly so marked in comparison to the lower temperature treatments. There was a significant reduction in the number of Golgi bodies, perhaps accounting for the substantial decrease in cell wall thickness. Throughout this study, a consistent negative correlation has been observed between cell wall thickness and increased growing temperature, but dimensions of the cuticle itself were not obviously different, however, from that observed in the three previous temperature treatments.

(ii) Mutant and Chemical Effects on Epidermal Cells

Plate 1.7 a,b & c illustrates the epidermal cell fine structure for the non-waxy (gl_3) mutant grown at 24/19 °C. Structurally the cytoplasmic layer differed from the waxy species in that there were fewer Golgi bodies, vesicles and endoplasmic reticulum. The plasmolemma, although continuous, was very irregular in outline and lay adjacent to a markedly hyaline region of the cell wall. The presence of densely stained globules throughout the cytoplasm, and particularly confined to the plasmolemma, were quite characteristic of the non-waxy mutant epidermal cells. Structurally the cell wall and cuticle were similar to those of the waxy control plants although the mutant cell walls appeared somewhat thicker.

Plates 1.8 a,b & c and 1.9 a,b & c illustrate the effects of herbicide treatment, applied to the roots, on the fine structure of epidermal cells. Plate 1.4 a,b & c should be referred to for comparison as representing the normal untreated 24/19 °C plants.

The appearance of the cytoplasm was similar to that observed for the controls, though some degradation of individual organelles was indicated. Ribosomal frequency in the dalapon treatment was higher while the amount of endoplasmic reticulum was marginally reduced. Similar differences were not noticeably apparent in the T.C.A. treated plants.

Frequent observations were a loss of plasmolemma integrity accompanied by a significant decrease in the frequency of vesicles for both herbicide treatments.

The dalapon treatment caused the hyaline layer external to the plasmolemma to be reduced and increased significantly in the T.C.A. treatment, although in both treatments, the cell wall thickness was significantly reduced, as was the thickness of the cuticles, particularly in the dalapon treated plants.

(iii) Fine Structure of Outer Epidermal Cell Wall

Plate 1.10a illustrates higher magnification detail of epicuticular wax (W), cuticle (Cu), cell wall (CW) and cytoplasm (Cy) for a 15/10 °C grown leaf in cross section. The structure of the surface wax resembled that observed elsewhere in replica studies. Structures which could be interpreted as non-membrane bound microchannels, ~ 7 nm in diameter, were evident within the cuticle (Plate 1.10b,c).

These microchannels (Mc) were found largely in the outer regions of the cuticle and lay at varying angles to the leaf surface. Occasionally microchannels of the type observed in *Plantago major* by Fisher & Bayer (1972) were observed to traverse the entire width of the cuticle (Plate 1.10d). The so-called microchannels at least cannot be sectioning artifacts since they were observed in sections cut from blocks at several different angles. Moreover, sections were always floated on water and never expanded with any organic solvent, so that stretching of sections could not be proposed to account for these structures either.

However, the microchannel system was never found to extend into the cell wall region, which consisted of closely packed microfibrils oriented parallel to the leaf surface giving a regular lamellate appearance. An

open mesh-like region of hyaline appearance in electron micrographs (C_N) was observed adjacent to the plasmolemma, which would probably represent a zone containing cell wall precursors of various kinds.

Plate 1.11a illustrates a cuticular thickening (C_T) frequently observed on the surface above the junction of adjacent epidermal cells. These structures were seen to consist of a loose fabric of materials having differential staining properties. The impression gained was of a network of fibrils, spaces and matrix materials more reminiscent of cuticles in the very earliest stages of formation in the bud. The appearance of such concentrations above cell margins accords with the earlier suggestion that during exposure new wall materials, presumably including cuticular components, are added principally at the cell margins with very little extension growth and accretion of wall materials occurring in the middle of outer tangential walls.

At higher magnification (Plate 1.11b) the orientation of fibrils, spaces, and/or microchannels, was seen to be quite irregular, though it was possible to imagine two types of orientation of structure taking place — one predominantly tangential and the other more radial. When sectioned parallel to the leaf surface as in Plate 1.11c, the material of low electron density representing interconnected network of spaces or possibly of occluded fibrils (M_E) was again clearly seen. Also of interest were structures (P) which appeared rounded in section and densely-stained, having approximately the same diameter (7 nm) as the individual strands of the fibrillar network (M_E). It was not possible, however, to observe connections between the fibrils or spaces and the rounded structures in these preparations.

The series of Plates 1.12 a - e illustrate in detail features of the cuticle and outer epidermal wall when sectioned parallel to the leaf surface at the levels indicated in Plate 1.12.

Plate 1.12a illustrates where a section has been cut from the outermost region of the cuticle, representing section line A in Plate 1.12. This area of sectioned cuticle is seen to be surrounded by sectioned epicuticular wax structures. The cuticular region is observed to contain darkly stained pore-like areas (P) ~ 7 nm in diameter (compare with Plate 1.11c).

Plate 1.12b illustrates a section cut further into the cuticular region (Plate 1.12, section line B). Where this section has cut the very

outer limits of the irregular cuticular surface, pore-like areas (P) are again observed. Grouped areas of microchannels (Mc) each measuring ~ 7 nm across may be observed where the section has apparently cut just beneath the cuticular surface. It is suggested that these channels might terminate at the cuticular surface as a pore from which wax finally exudes onto the leaf surface.

A section cut obliquely across the surface (Plate 1.12c) shows wax (W), cuticle (Cu), and cell wall (CW) regions. The more darkly stained pore-like areas (P) noted previously were observed in the outer regions of the cuticle. No definite demarcation between cuticle and cell wall was evident, the two being seen to merge irregularly. No well-developed pectin layer was evident in this transition zone. The cell wall (CW) consisted of irregularly shaped subunits which frequently appeared hexagonal in outline.

Plate 1.12d illustrates a section of cell wall cut parallel to the leaf surface as indicated by Plate 1.12, section line D. The cellulose matrix of the cell wall was observed to consist of a heterogeneous array of subunits which exhibited a variable banded staining pattern.

Plate 1.12e illustrates a section from the junction of the inner cell wall and the plasmolemma, cut at the level indicated in Plate 1.12, section line E. The cell wall (CW) is found to merge into areas of cell wall synthesis (C_N) adjacent to which lies the plasmolemma (Pl). Within the cytoplasm (Cy) are ribosomes and an extensive array of microtubules (M) which generally have one end of their length in the vicinity of the cellulose area (C_N). With this and similar evidence (e.g. Newcomb, 1969), it seems likely that microtubules may have a function in primary wall formation whereby cellulose microfibrils may be oriented parallel to one another during formation and deposition.

(iv) Effect of Growing Temperature on Fine Structure of Wax

Plate 1.13a illustrates the surface wax characteristic of a plant grown at 15/10 °C. Wax rods of average length 1.8 μm, and rarely exceeding 3.4 μm with a base diameter of 0.2 - 0.6 μm typify this condition, and platelet type wax was consistently absent. The same wax, viewed with a scanning electron microscope, is presented in Plate 1.13a (inset). Wax rods have been shown to have a hollow centre (Johnson & Jeffree, 1970) but the use of the term 'tube' which is commonly found in the literature has been avoided in this study. While technically correct

for the structures as they appear in replica form, the term is confusing when applied to the interpretation of what has been replicated on the leaf surface itself.

Plate 1.13b illustrates the surface was characteristic of a plant grown at 18/13 °C. Wax rods of the type observed in Plate 1.13a may be seen again in conjunction with a series of narrow flat wax platelets. Quantitatively the ratio of rods:platelets was approximately 2:1.

Plate 1.13c illustrates the surface wax characteristics of a plant grown at 21/16 °C. In contrast to the previous transition temperature (18/13 °C) there was here a predominance of complex narrowly branched open platelets. These generally occurred as a single layer lying ~ 0.3 μm above and parallel to the cuticle surface. Wax rods were still present but in lesser amounts.

Plate 1.14a illustrates the surface wax characteristic of a plant grown at 24/19 °C. The wax platelets were more complex than those observed in the previous temperature (21/16 °C) with fusion of the primary branching being quite conspicuous. The platelets form an elevated platform ~ 0.3 μm above the leaf surface. Wax rods were not uncommon.

Plates 1.14b,c and 1.15a,b illustrate the surface wax characteristics of plants grown at 27/22 °C, 30/25 °C, 33/28 °C and 36/31 °C respectively. In Plate 1.15b (inset) 36/31 °C wax as viewed by a scanning electron microscope is presented. A positive correlation between increasing growing temperature and wax platelet complexity was observed. Fusion of the primary and secondary branches became so pronounced with increasing temperature that completely solid wax platforms were evident at the higher temperatures. In general, only the outer periphery of such wax platforms showed the typical branching pattern. The presence of wax rods was quite rare in these higher temperatures.

(v) Mutant and Chemical Effects on Wax Fine Structure

Plate 1.15c illustrates the surface wax characteristic of the non-waxy (gl_3) mutant grown at 24/19 °C. In contrast to the waxy plant, the wax was present merely as a smooth largely non-structural layer.

Plate 1.16a,b illustrates the surface characteristics of plants treated with T.C.A. and dalapon respectively. T.C.A. had the effect of

quantitatively reducing wax formation to a significant degree although qualitatively the wax still resembled that of the control (24/19 °C, Plate 1.16c).

Dalapon, as well as reducing wax formation quantitatively, altered the wax qualitatively. The platelet wax formation was almost completely suppressed while the rod wax formation was reduced to rather short irregularly-shaped wax blocks.

Plate 1.17a illustrates the surface wax characteristic of a 24/19 °C grown expanding leaf just prior to two root applications of 2.5×10^{-4} moles of T.C.A. over a two-day period. Plate 1.17b illustrates the surface of the same leaf as the control (1.17a) twenty-four hours after the last herbicide application. An almost complete dewaxing effect, removing all the wax originally present, was clearly apparent. The only structural wax then present was that characteristic of a plant treated with T.C.A. for some duration (Plate 1.16a). Leaves that had fully expanded prior to the herbicide treatment showed little, if any, tendency to being dewaxed (Plate 1.17c).

(vi) Effect of Rapid Temperature Changes on Wax Fine Structure

Plate 1.18a,b represents the 15/10 °C and 36/31 °C leaf surface wax controls respectively.

Plates 1.19 - 1.22 illustrate a series of leaf surface wax modifications apparent when a 15/10 °C grown plant was transferred to a 36/31 °C temperature regime.

Plate 1.19a. Wax modification was evident within six hours and took the form of the existing wax rods branching out at ~ 90° to their vertical axes, though fusion into wax platelets was not apparent. Many of the original wax rods established during the 15/10 °C temperature regime were consistently observed to have been lost following a low to high temperature transition.

Plate 1.19b. Within twenty-four hours after the transfer, the formation of wax platelets, characteristic of high temperatures, had begun, while branching of some of the original wax rods was still continuing.

Plate 1.19c. Forty-eight hours after the transfer, the frequency of wax rods was significantly reduced while the formation of complex overlapping platelets was very conspicuous.

Plate 1.20a. Seventy-two hours after the transfer, the wax platelet formation became even more complex. They became more obviously fused and markedly increased in coverage of the leaf surface.

Plate 1.20b. Ninety-six hours after the transfer, the modification of the wax was so significant that the wax pattern virtually appeared the same as a 36/31 °C control leaf (Plate 1.18b).

Plate 1.20c. One hundred and twenty hours after the transfer, the wax modification appeared to be complete, resembling that of a 36/31 °C control in all respects. No further modification was apparent after this period.

The above results brought about by a direct transfer from 15/10 °C to 36/31 °C conditions brought into question the physiological shock caused to the plant by such a sudden drastic change. Accordingly, it was decided to programme a gradual increase from the 15 °C to the 36 °C temperature regime to allow for adaptation.

Plate 1.21a illustrates the wax modification on the leaf surface of a 15/10 °C grown plant resulting from a programmed temperature increase at the rate of 0.5 °C per hour for forty-two hours (21 °C range) to 36 °C. Structurally the wax layer appeared to consist of a massive growth of long narrow single stranded ribbons, with very little evidence of the usual upright wax rods. This enormous proliferation of wax due to the gradual increase in temperature bore little resemblance to the wax as seen forty-eight hours after a direct transfer from low to high temperature (Plate 1.19c). In the light of this comparison, it is apparent that in fact the physiological shock of moving the plant directly to a 21 °C higher temperature environment had a less stimulatory effect on wax modification than a steadily increasing temperature.

After growing plants for a substantial period at 15/10 °C, transferring to the higher temperature, whether suddenly or more gradually, placed the plants under severe stress. The leaf area and thickness for the 15/10 °C grown plants was large when compared to the 36/31 °C adapted plants, while the temperature increment for a transferred plant was considerable. In an attempt to further ascertain the sensitivity in regard to modification of the leaf wax to increased temperature, a temperature increment of 12 °C was employed instead of 21 °C. Again, wax modified by a direct and programmed temperature increase were compared by transferring 15/10 °C grown plants to 27/22 °C.

Plates 1.21b and 1.21c illustrate a direct and programmed temperature transition to 27/22 °C over a twenty-four hour period respectively. The programming rate for the latter was 0.5 °C per hour. The wax modification for the two treatments is qualitatively similar, consisting of initial wax platelet formation and branching of the pre-existing wax structures. The programmed treatment has significantly more wax coverage as noted previously where the temperature increment was 21 °C (Plates 1.19c and 1.21a).

Plate 1.22a,b illustrates the wax observed in thin section of a plant having undergone a direct transition from 15/10 °C to 27/22 °C for twenty-four hours. The observed wax modification confirmed the results obtained in carbon replica studies taking the form of branching wax rods and platelet formation. Plate 1.22c illustrates the upright typically non-branched wax rod structure of a 15/10 °C control.

Attempts to obtain a wax modification in the form of wax rods by transferring a 36/31 °C temperature grown plant to a 15/10 °C temperature regime were completely unsuccessful. The only alteration of the wax surface even after a five-day period at 15/10 °C was a partial loss of the pre-existing wax platelets (Plate 1.23a). Plates 1.23b and 1.23c represent a 36/31 °C and 15/10 °C control respectively.

DISCUSSION

Variation in growing temperature has been shown to produce an effect on the cytoplasm and leaf structure generally, while the modification of the surface wax pattern was quite remarkable. While these variations are of interest in their own right, no specific correlation was possible using conventional electron microscopy.

Structurally, the non-waxy (gl_3) mutant plant did not differ significantly from the normal waxy plant, in spite of having substantially reduced wax production. In this and previous studies (Juniper, 1959a; Dewey *et al.*, 1961), herbicidal treatments have been found to reduce wax production and modify the wax pattern on the leaf surface. Fine structural similarities were seen between the wax herbicidal and high temperature treatments, but these did not correlate with wax production, which was high at high temperature and low with herbicides. Suppression of wax formation, whether due to a genetic mutation or to herbicide treatment, would seem to be specifically related to a biochemical block in the wax synthesising pathway (Kolattukudy, 1965;

Macey & Barber, 1970a,b), while wax chemistry and/or physical factors were the cause of the variations in fine structure of wax due to different temperatures.

Large vesicles, possibly lipoidal in nature, were frequently observed in association with Golgi bodies. Hallam (1970b) has observed a similar relationship in *Eucalyptus* and noted an apparent migration of the Golgi in the direction of the cell wall. If such a migration occurred, the vesicles may have been responsible for the liberation of wax precursors across the plasmolemma into the cell wall. The application of an autoradiographic study would have to be employed to confirm this suggestion.

The ability of a *Brassica* plant to begin modifying its structural wax on the leaf surface in as little as six hours and significantly to that characteristic of a higher temperature environment in less than one hundred hours has been readily demonstrated. Carbon replica and thin section techniques have shown that the qualitative alteration of a rod type wax to plates was achieved in part by the modification of pre-existing wax.

The rapid wax generation response brought about by transfers to higher temperatures was apparently due to the response of an environmental protective mechanism. No cell structural differences attributable to the alteration of the wax pattern were evident. It is suggested that increased growing temperature affected wax production at the biochemical level, causing a rapid biosynthesis and subsequent movement of fresh wax to the leaf surface. Extrusion of wax already synthesised and present in the cell wall-cuticle complex, or a greater dispersal of a non-crystallised wax solvent at the leaf surface, are considered to be of little importance in bringing about a wax modification.

Theoretically it remains possible that transition to a higher temperature regime could stimulate the production of a wax solvent alone on the leaf surface, this in turn redissolving the existing wax. A secondary effect of the higher temperature would then have been to recrystallise the wax in the characteristic pattern observed. The practical aspects of such a system seem unnecessarily complex and unlikely.

Conceivably wax modification caused by increased wax production could be stimulated by a series of temperature-sensitive enzymes which are progressively activated by increasing temperature. If such a system did operate, it is apparent that once a particular enzyme was activated, the lower temperature enzymes might be deactivated since low to high temperature wax modifications were quite irreversible. This would seem to be not an unreal situation since decreasing the growing temperature would not pose the physiological stresses of a temperature increase.

A sudden application of trichloroacetic acid to the root system of an established plant has been observed to significantly dewax the expanding waxy leaves already present. This effect can be achieved so rapidly that it is tempting to suggest that the cuticle-surface wax zone may be altered such that the retention of the original wax layer is lost. This alteration may be achieved by the herbicide inhibiting an enzyme system which produces normal wax precursors but fails to inhibit and perhaps even enhances the production and subsequent flow of wax solvent to the leaf surface. This being so, an extrusion of wax solvent on to the leaf surface alone might dissolve the surface wax attachment zone, the wax in turn being lost from the leaf surface.

This reasoning may be extended to the observation that fully expanded leaves do not develop or regenerate large amounts of surface wax (Hallam, 1970c). The failure of a fully expanded leaf to become dewaxed by a sudden herbicide treatment could be explained by the cessation of the wax solvent flow in conjunction with the wax precursor synthesis.

The cessation of wax production in a fully expanded leaf may not be necessarily due to a discontinuation of wax precursor formation, but rather of the solvent which mobilises the wax to the leaf surface. If this were the case, the cell walls and possibly the cytoplasm may be heavily loaded with wax precursors up to the time of senescence.

Sectioning leaf tissue parallel and perpendicular to the leaf surface has confirmed the heterogeneous nature of both cell wall and cuticle. Wax or its immediate precursors are synthesised in the epidermal cell (Kolattukudy, 1970b), traverse the plasmolemma either actively or passively, and move through the cell wall. This study suggests that in *Brassica* at least, the movement through the cell wall is one of diffusion across a concentration gradient. Although not ascertained, the movement could be in the form of lipoid droplets through

the cell wall as suggested by Bolliger (1959). No evidence has been obtained for movement through specific channels within the cell wall as suggested by Hall (1967b).

It is suggested that subsequent movement across the cuticle in that region adjacent to the cell wall occurs primarily by non-localised diffusion. The cell wall and cutin meshwork would seem to present very little resistance to diffusion. Subsequent movement in the outer region of the cuticle presumably occurs through what appears to be a microchannel system. These microchannels are oriented at various angles to the leaf surface, one end of an individual channel generally being traceable to the cuticle surface. The outer zone of the cuticle appears to be a reticulate system of microchannels opening out on to an exceedingly irregular cuticle surface. The occurrence of microchannels completely traversing the cuticle appear to be too infrequent to solely support Hall's hypothesis (Hall, 1967a) that wax migrates through the entire cuticle via channels.

While the results indicate a combined diffusion-microchannel system for the excretion of leaf waxes in *Brassica*, this is not to say that in some plants migration does not occur by way of anastomosing platelets as suggested by Hallam (1964).

Inconclusive evidence from the various sectioning techniques employed suggest that the microchannels terminate at the cuticle surface in a random array of pore-like openings. Methods other than conventional electron microscopy would have to be employed to substantiate this observation.

Plates 1.1 - 1.23

The following plates illustrate the results for waxy *Brassica napus* plants unless specifically stated otherwise.

All thin sections were prepared from material fixed with glutaraldehyde/osmium tetroxide and stained with uranyl acetate and lead citrate.

Leaf surface wax preparations employed Pt/C shadowing and carbon replication, except in the case of scanning electron micrographs where carbon and gold/palladium coatings were employed.

Dimension lines on micrographs represent 1 μm except where indicated otherwise.

Plate 1.1: Light micrographs illustrating the upper epidermis and adjoining palisade cells of plants grown at:

(a) 15/10 °C

(b) 24/19 °C

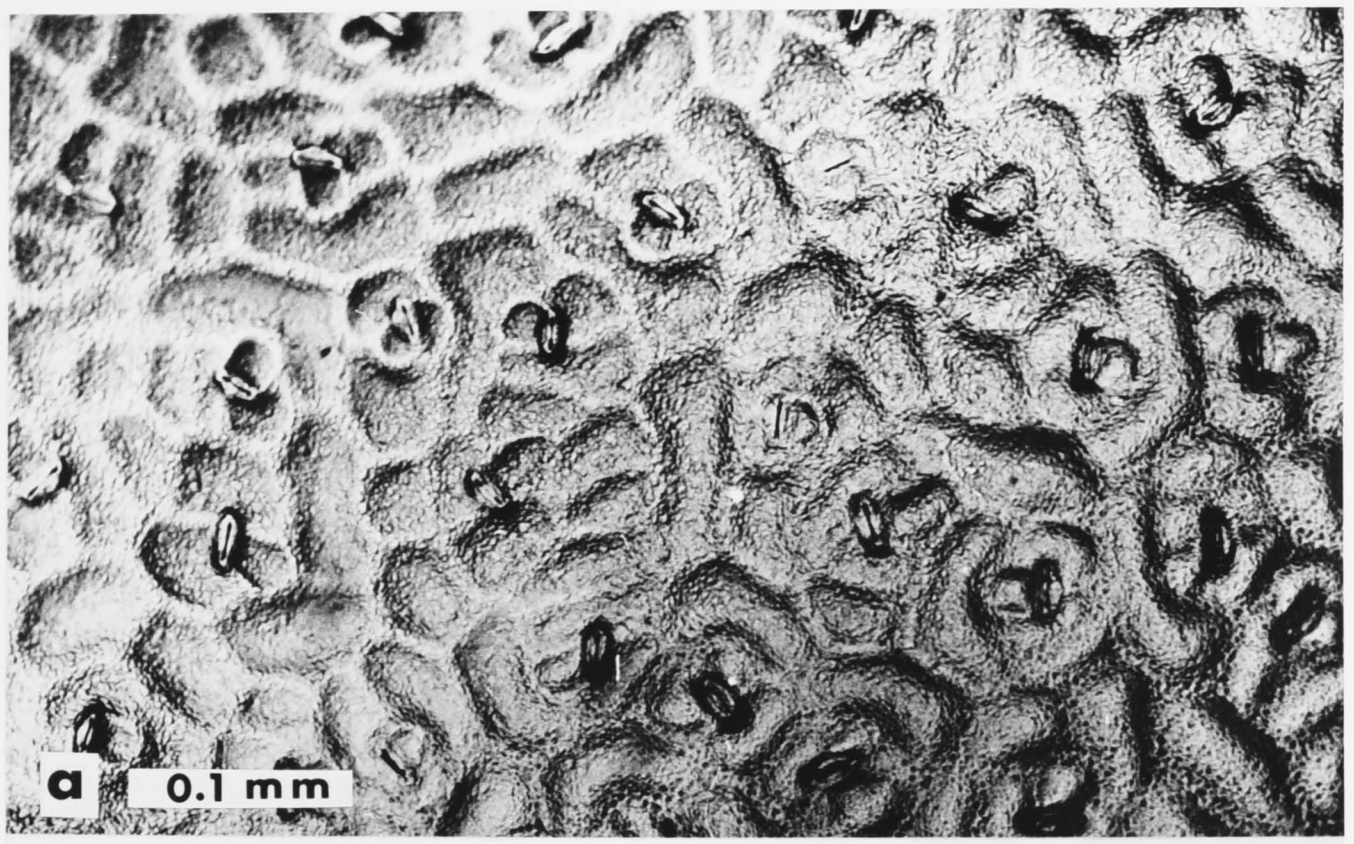
(c) 36/31 °C.

Plate 1.2: Light micrographs illustrating low power characteristics of the upper leaf surface for plants grown at:

(a) 15/10 °C

(b) 24/19 °C

(c) 36/31 °C.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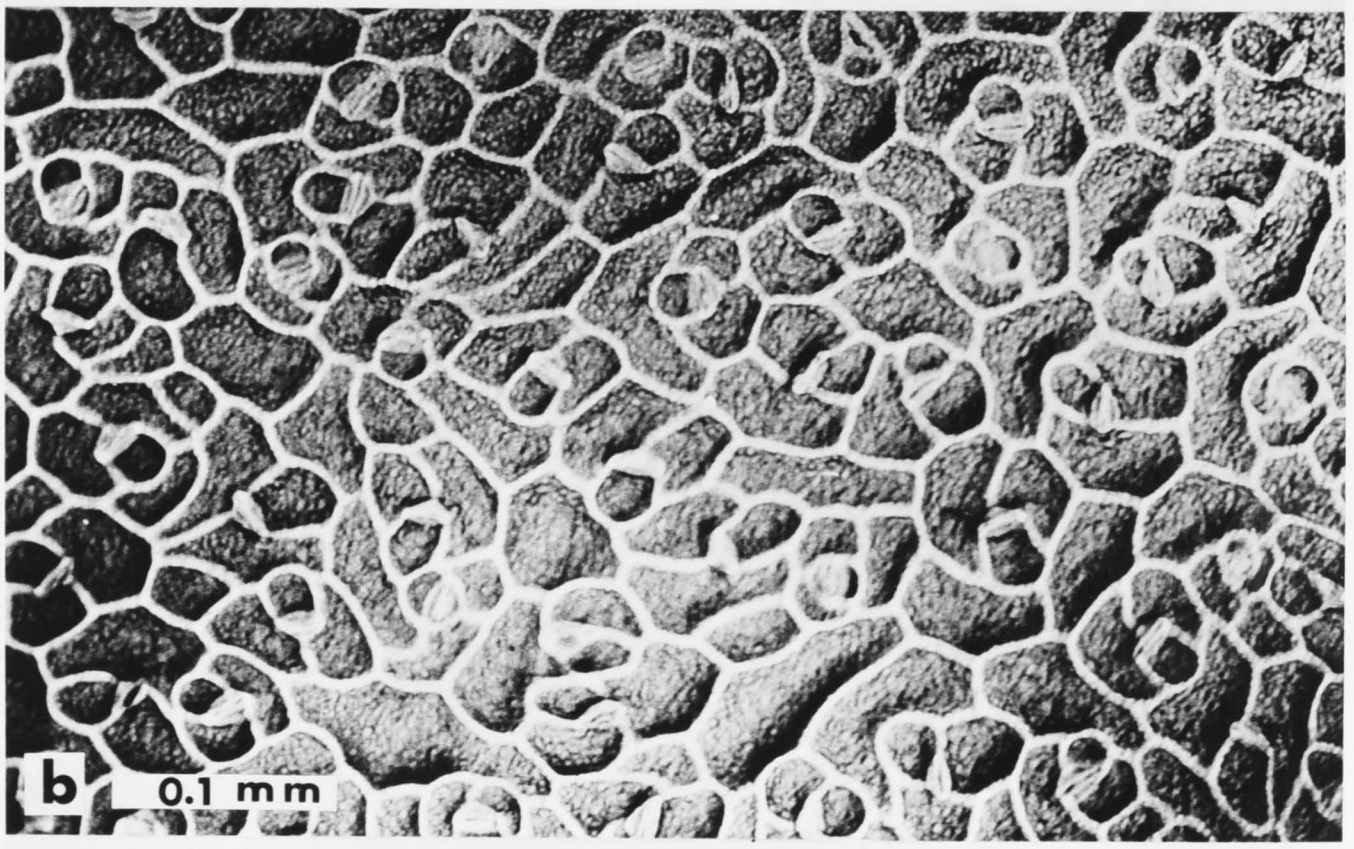
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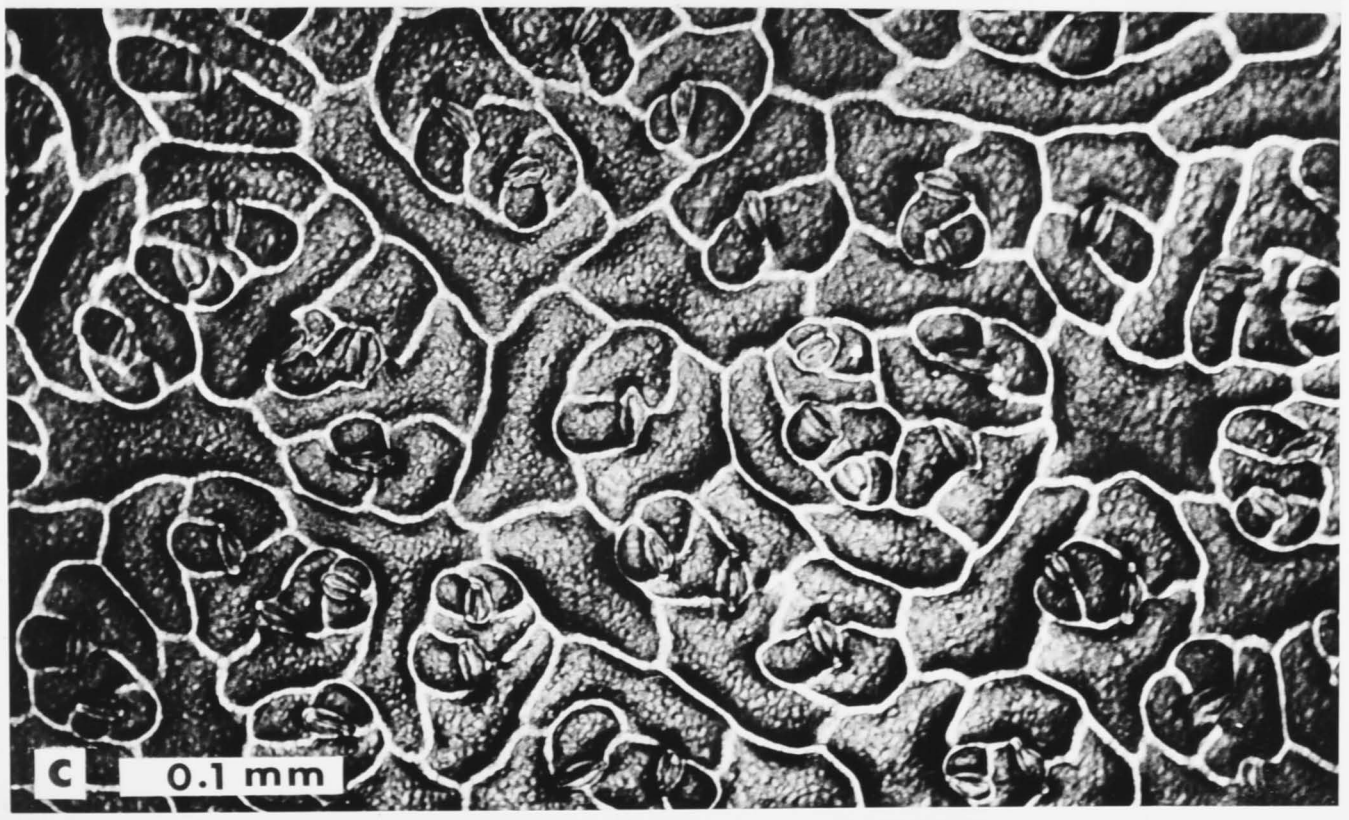
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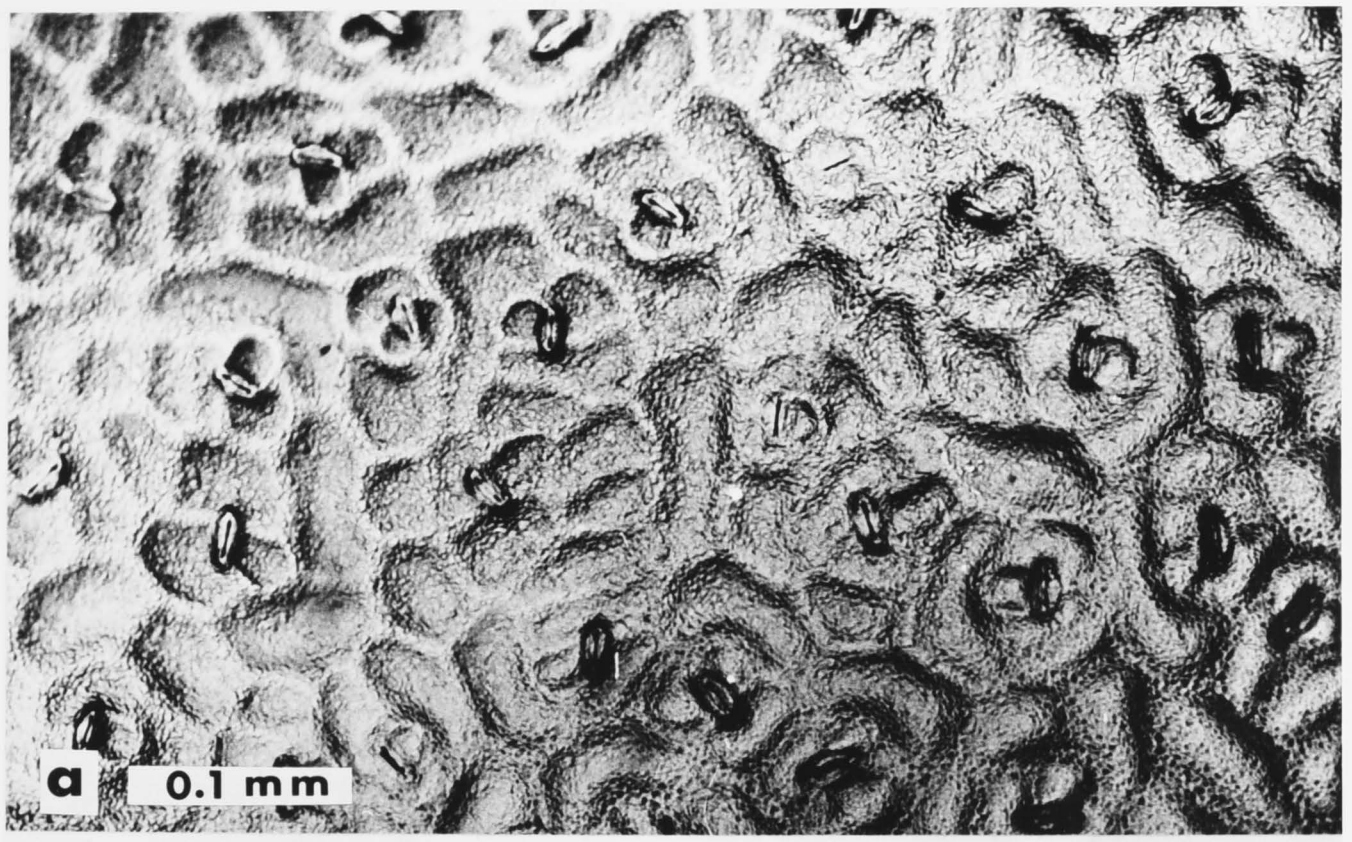
Plate 1.2: Light micrographs illustrating low power characteristics of the upper leaf surface for plants grown at:

(a) 15/10 °C

(b) 24/19 °C

(c) 36/31 °C.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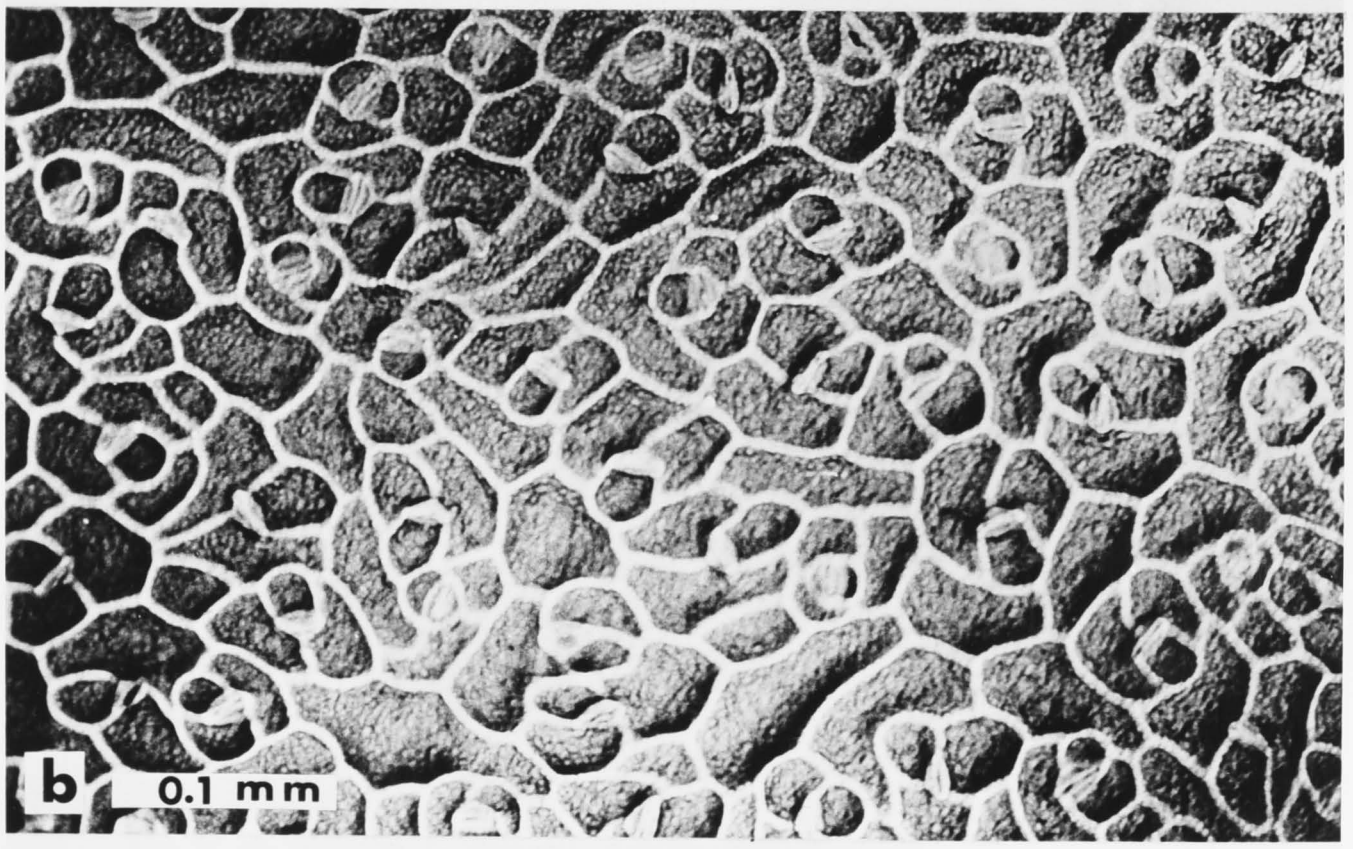
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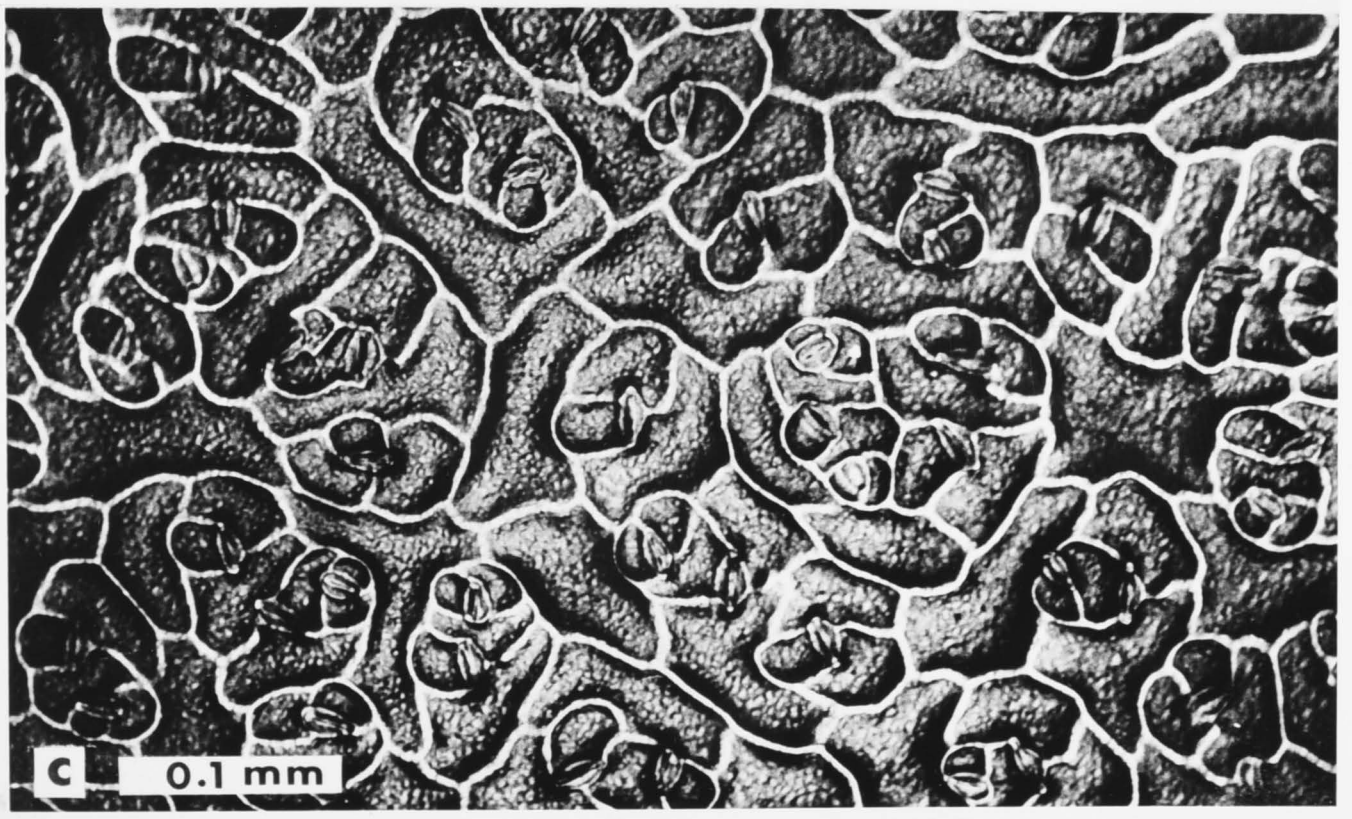
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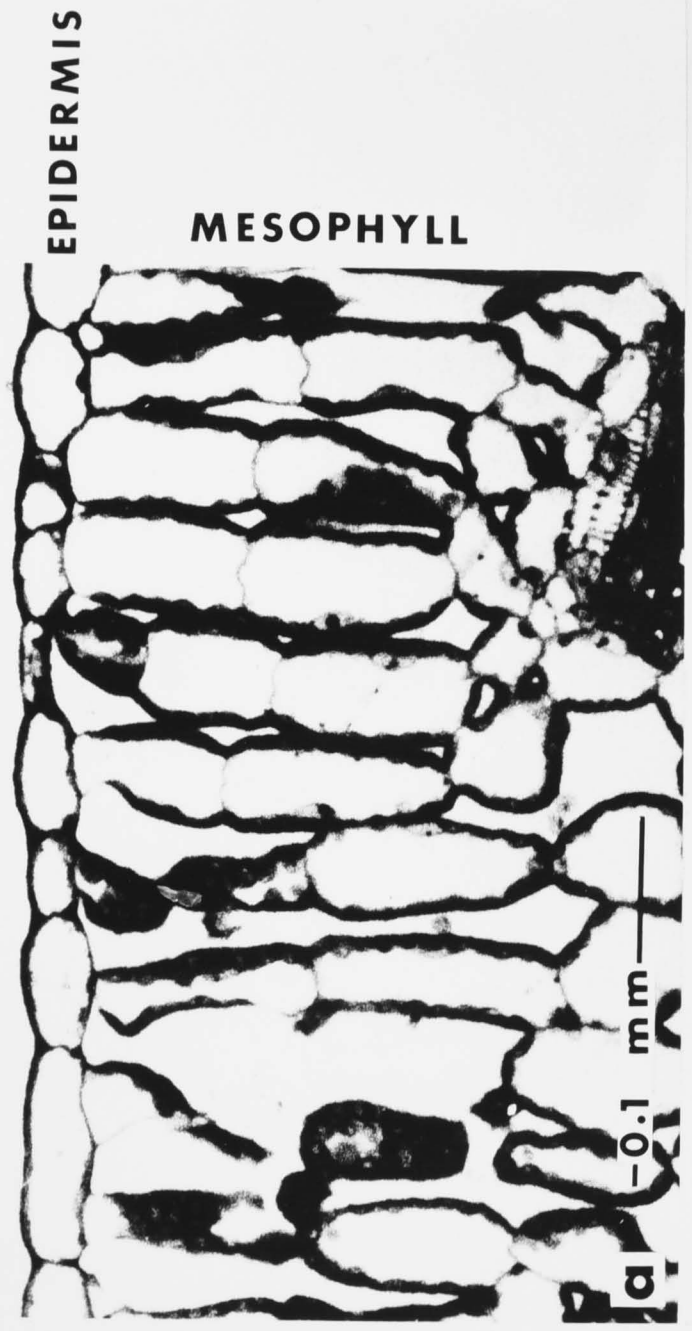
Plate 1.1: Light micrographs illustrating the upper epidermis and adjoining palisade cells of plants grown at:

(a) 15/10 °C

(b) 24/19 °C

(c) 36/31 °C.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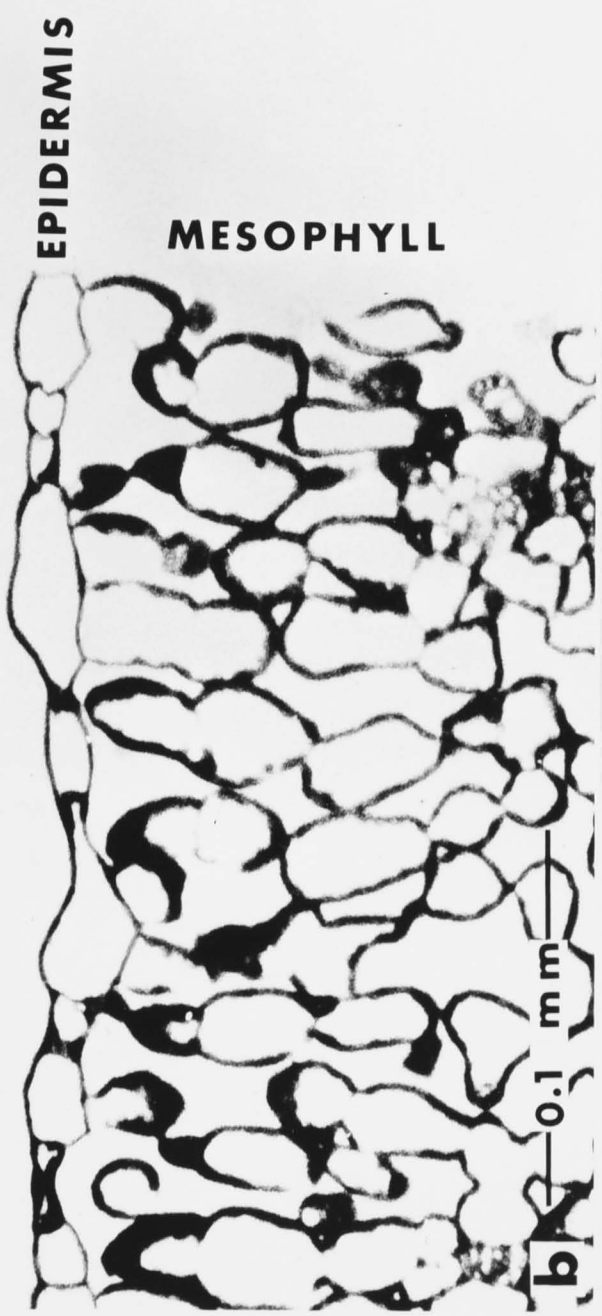
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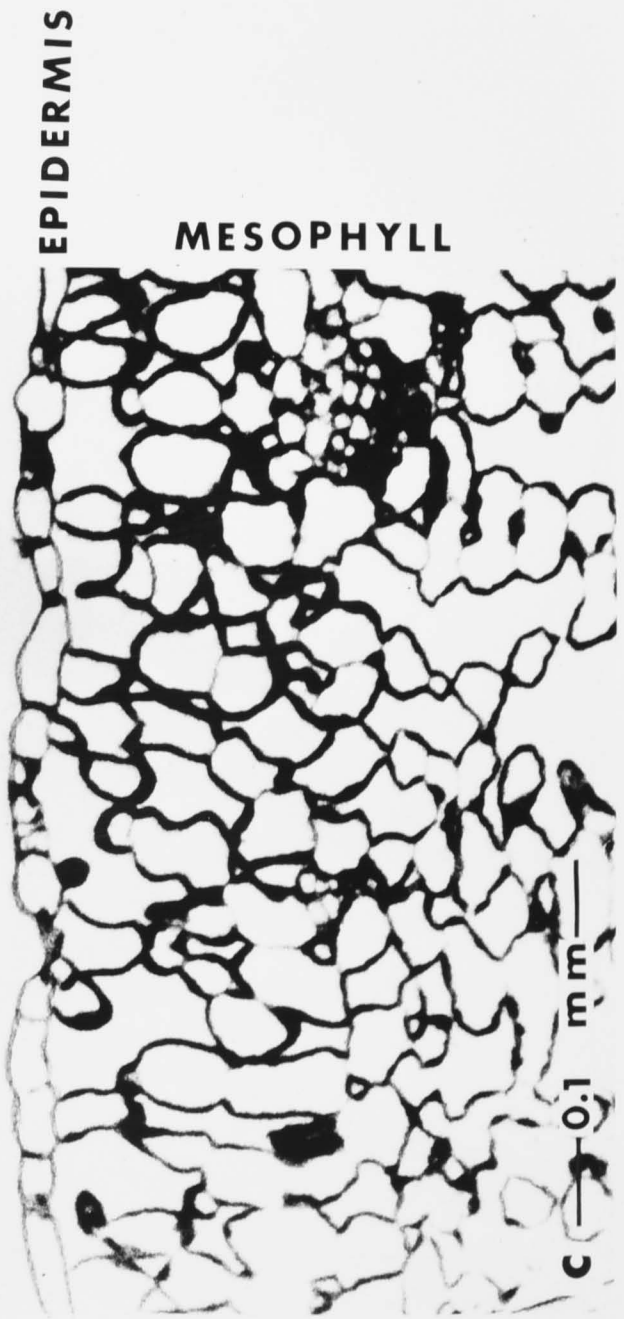
EPIDERMIS
MESOPHYLL
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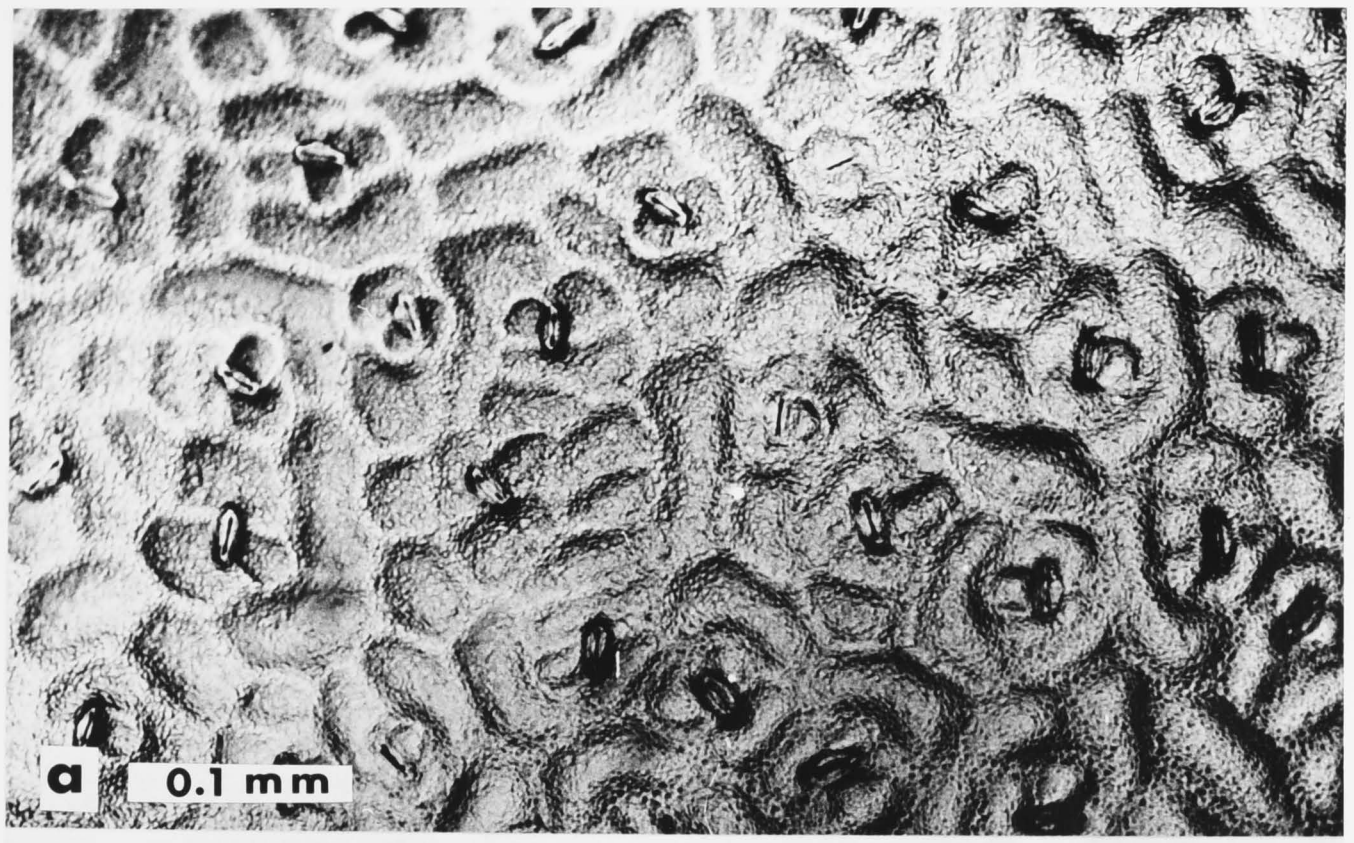
Plate 1.2: Light micrographs illustrating low power characteristics of the upper leaf surface for plants grown at:

(a) 15/10 °C

(b) 24/19 °C

(c) 36/31 °C.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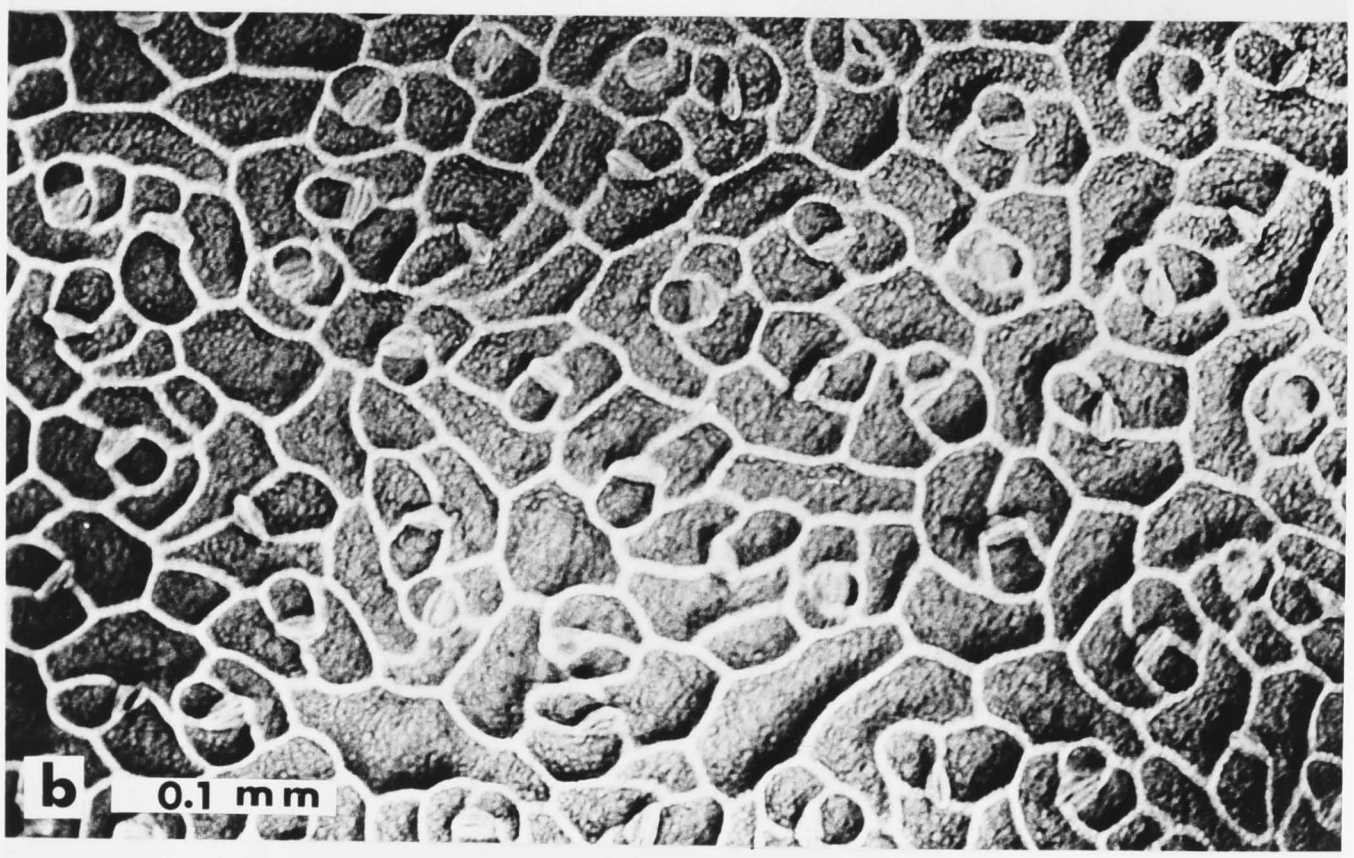
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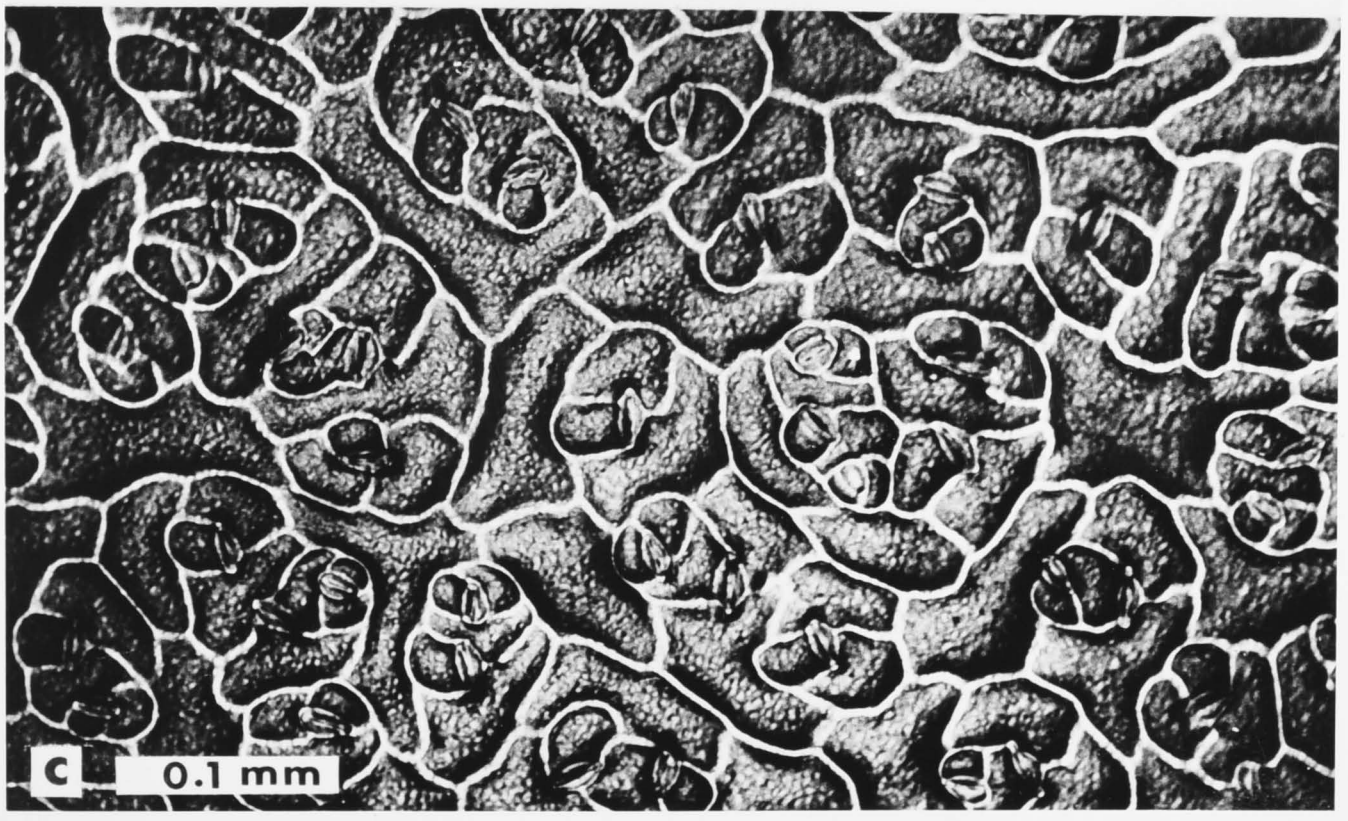
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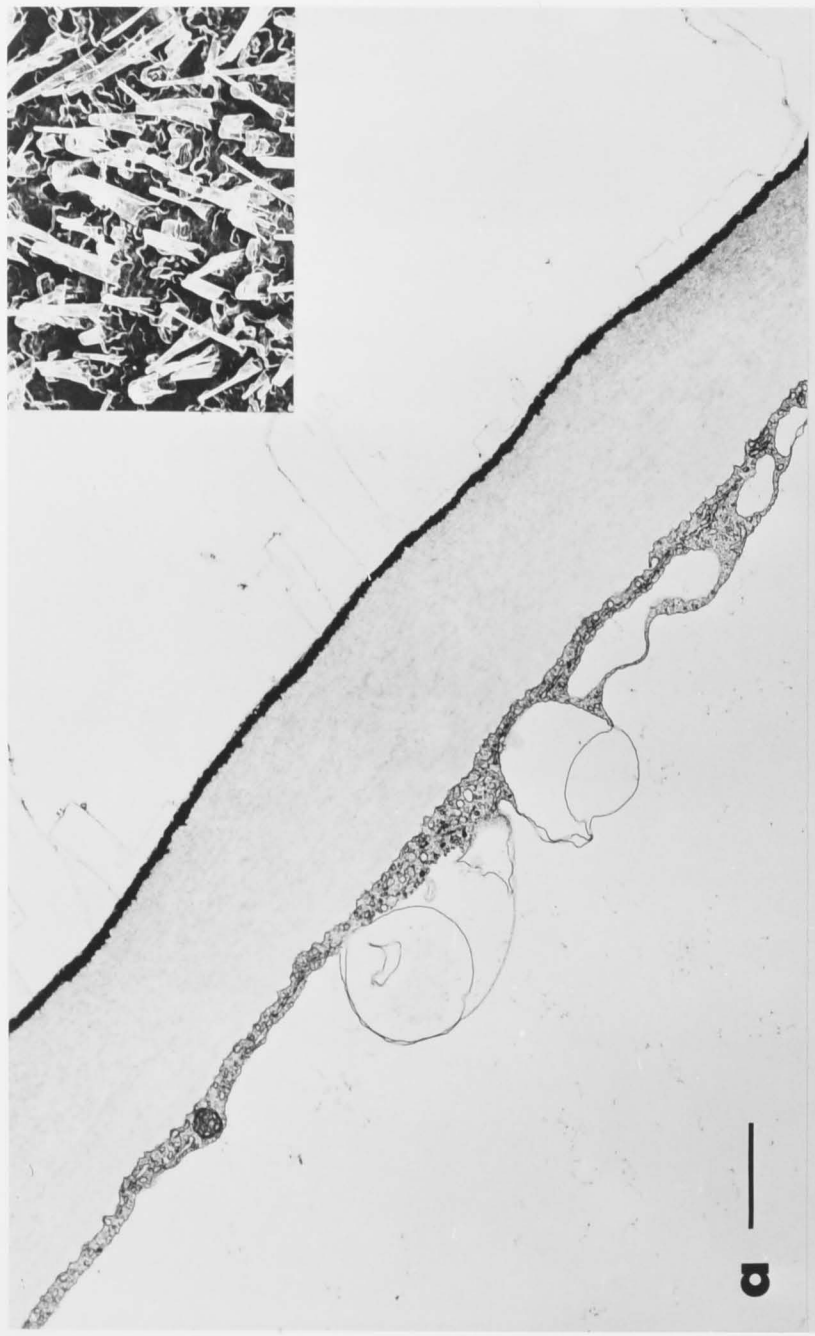
Plate 1.3 a,b,c: Electron micrographs illustrating the outer epidermal cell wall fine structure of 15/10 °C grown leaves. Inset illustrates surface wax characteristics.



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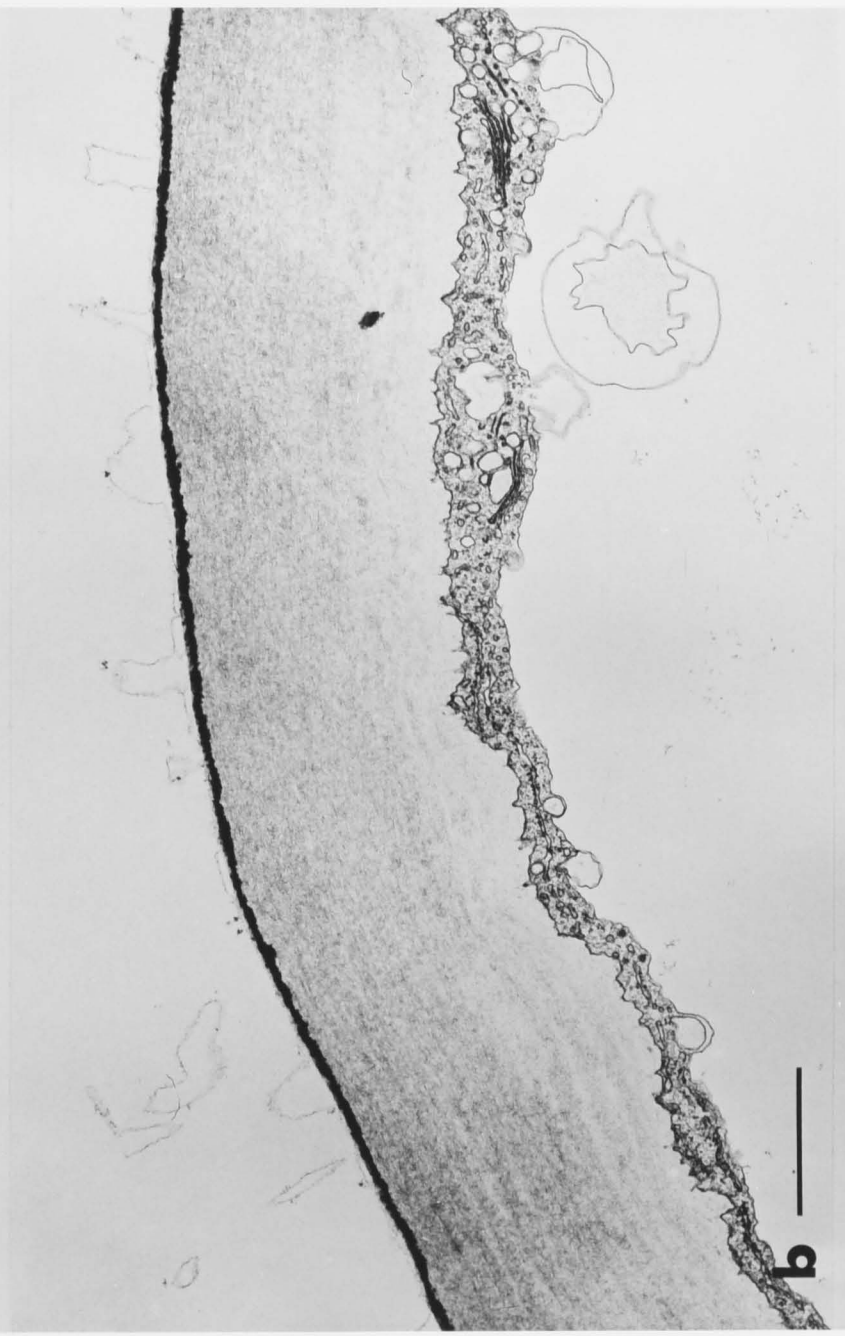

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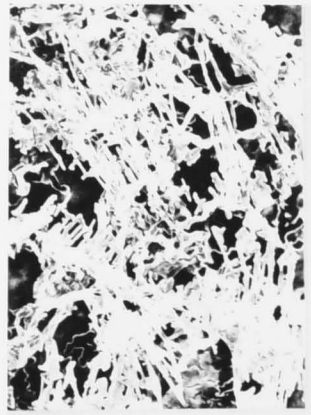
c

Plate 1.4 a,b,c: Electron micrographs illustrating the outer epidermal cell wall fine structure of 24/19 °C grown leaves. Inset illustrates surface wax characteristics.



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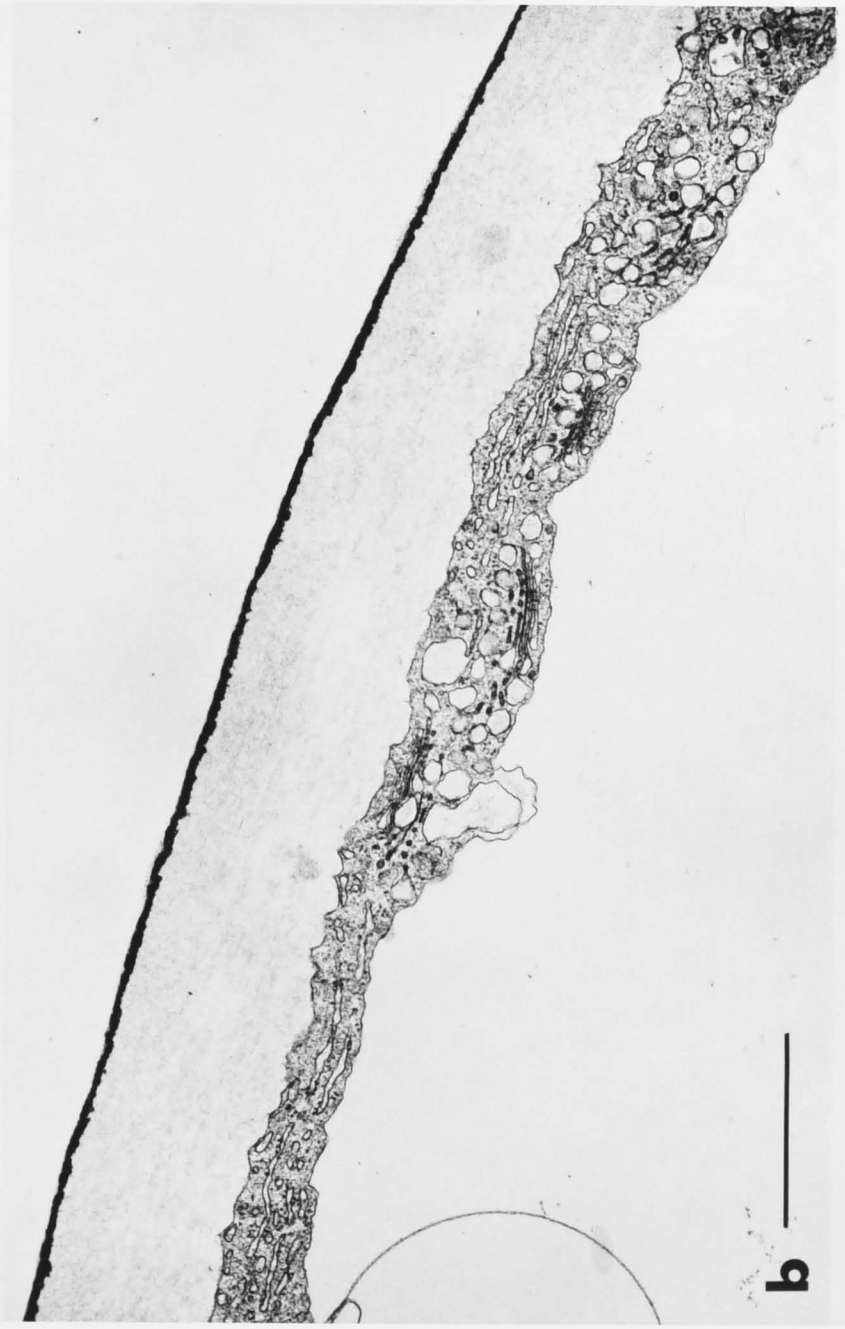

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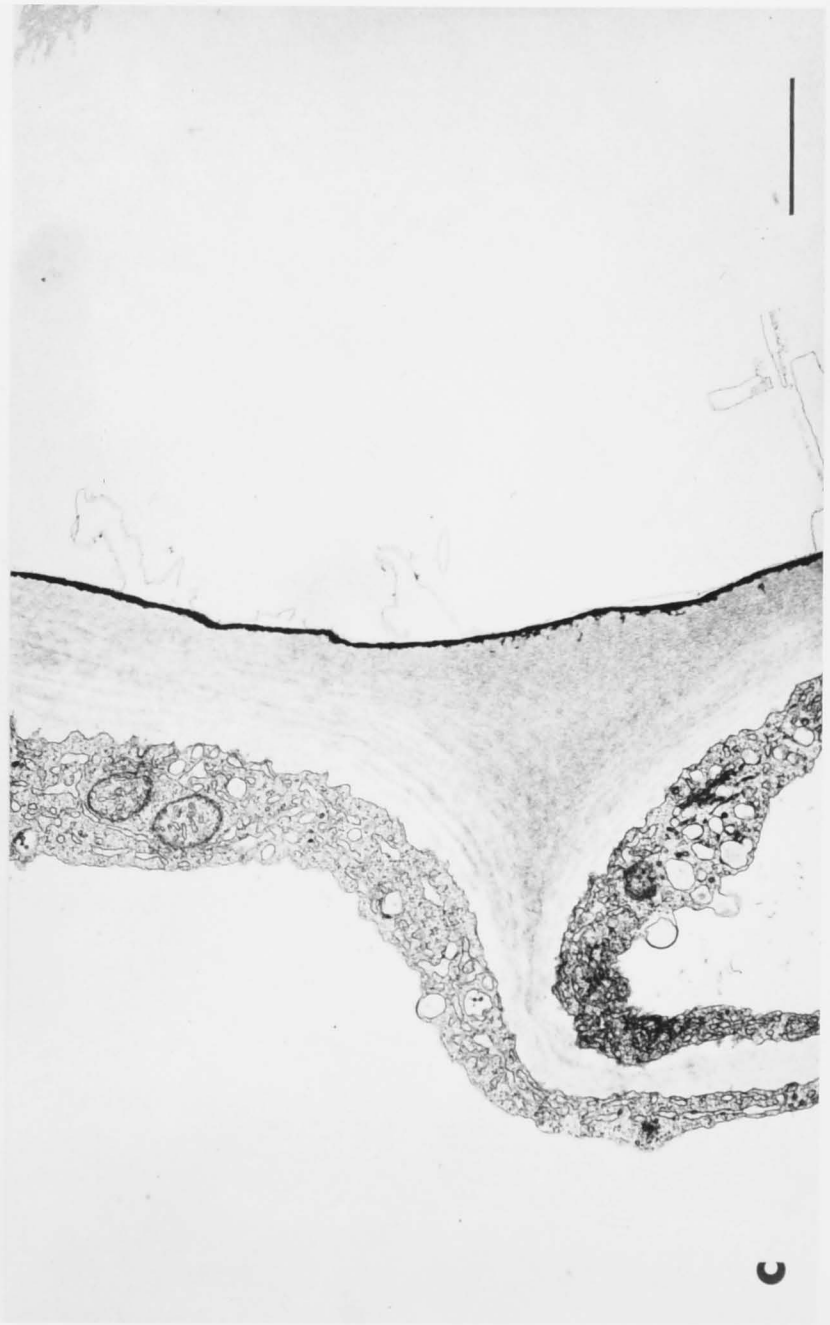

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Plate 1.5 a,b,c: Electron micrographs illustrating the outer epidermal cell wall fine structure of 27/22 °C grown leaves. Inset illustrates surface wax characteristics.

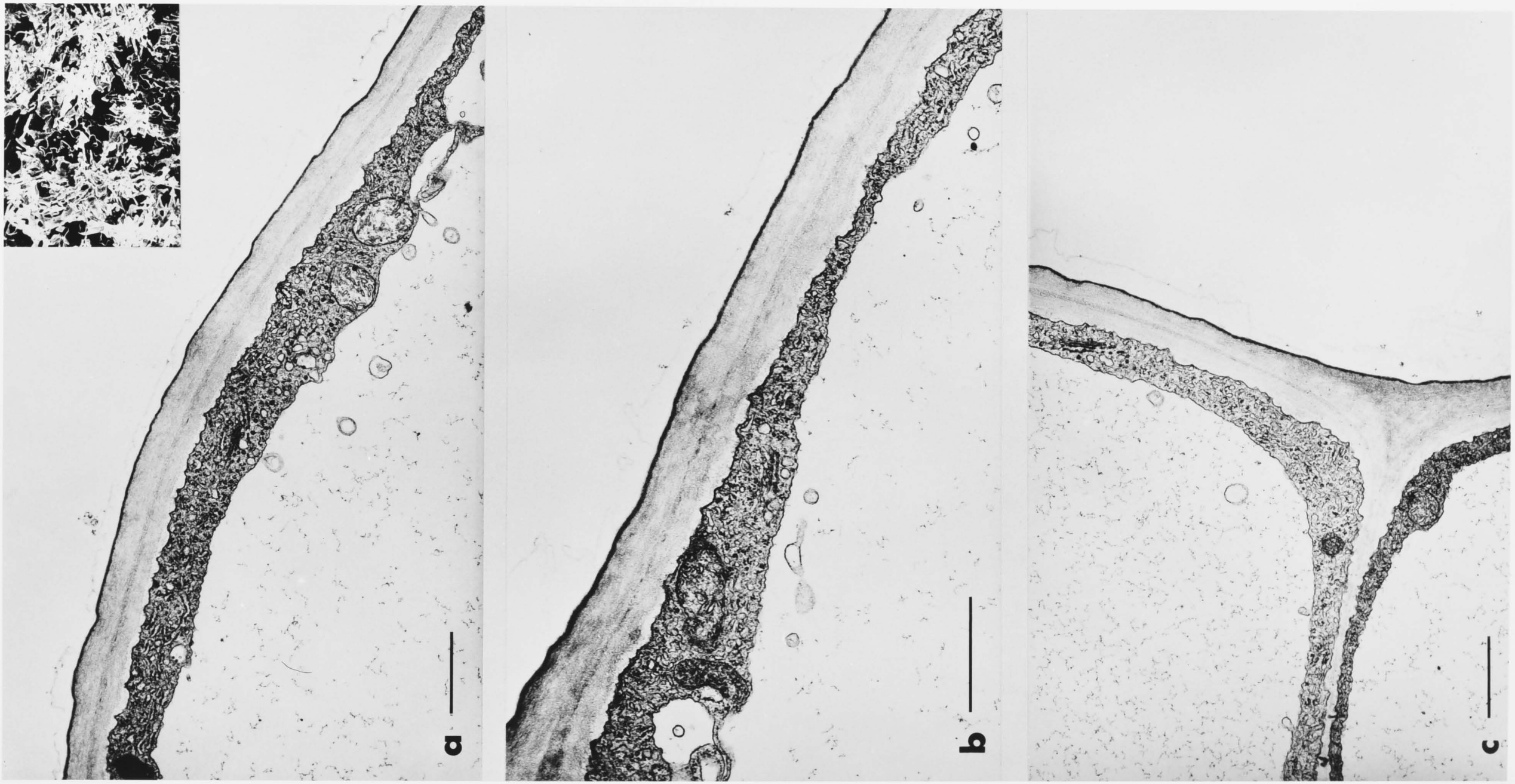
a
b
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Plate 1.6 a,b,c: Electron micrographs illustrating the outer epidermal cell wall fine structure of 36/31 °C grown leaves. Inset illustrates surface wax characteristics.

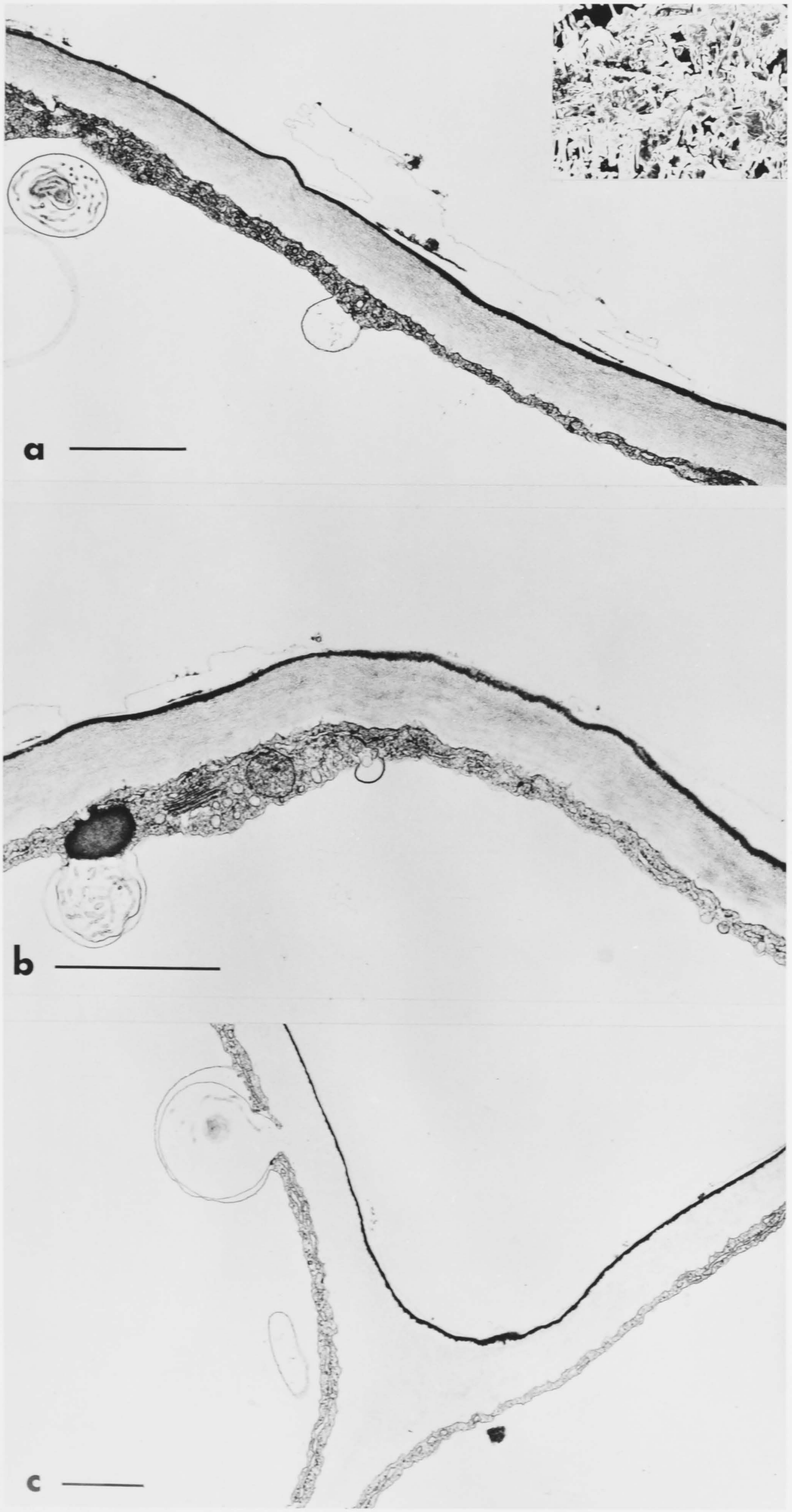
a
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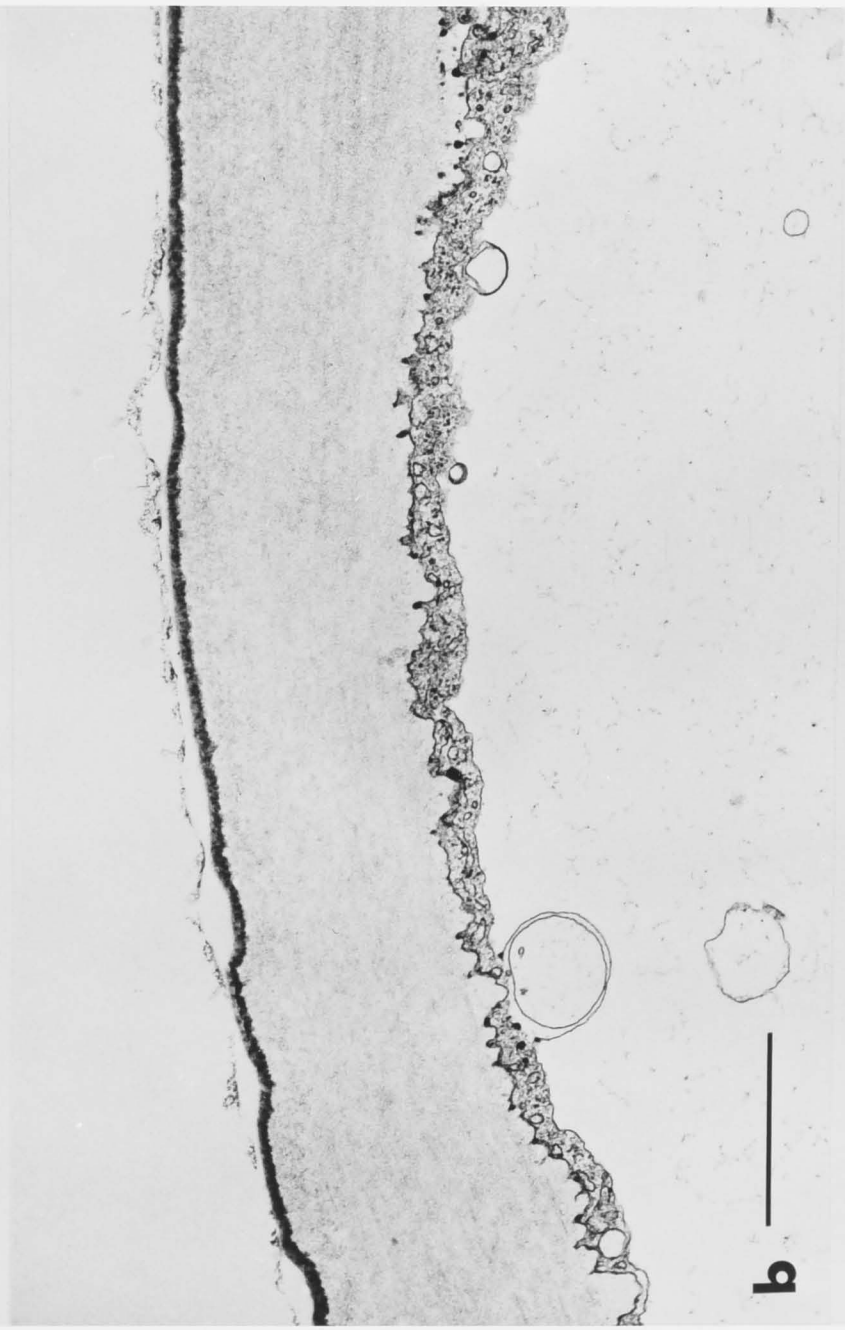
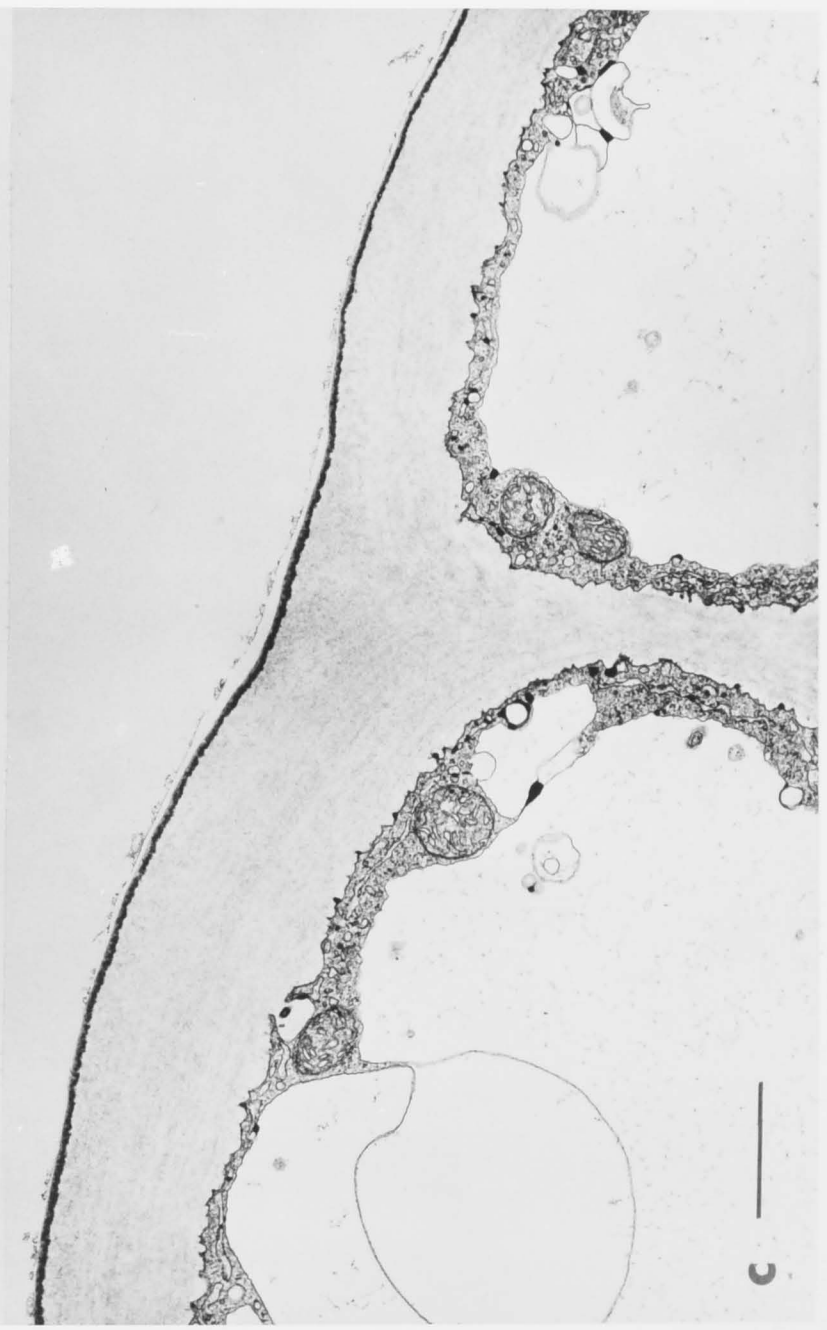
Plate 1.7 a,b,c: Electron micrographs illustrating the outer epidermal cell wall fine structure of non-waxy (gl_3) mutant leaves grown at 24/19 °C. Inset illustrates surface wax characteristics.

a



b



c

Plate 1.8 a,b,c: Electron micrographs illustrating the outer epidermal cell wall fine structure of 24/19 °C grown leaves after root applications of dalapon (2,2-dichloropropionic acid). Inset illustrates surface wax characteristics.

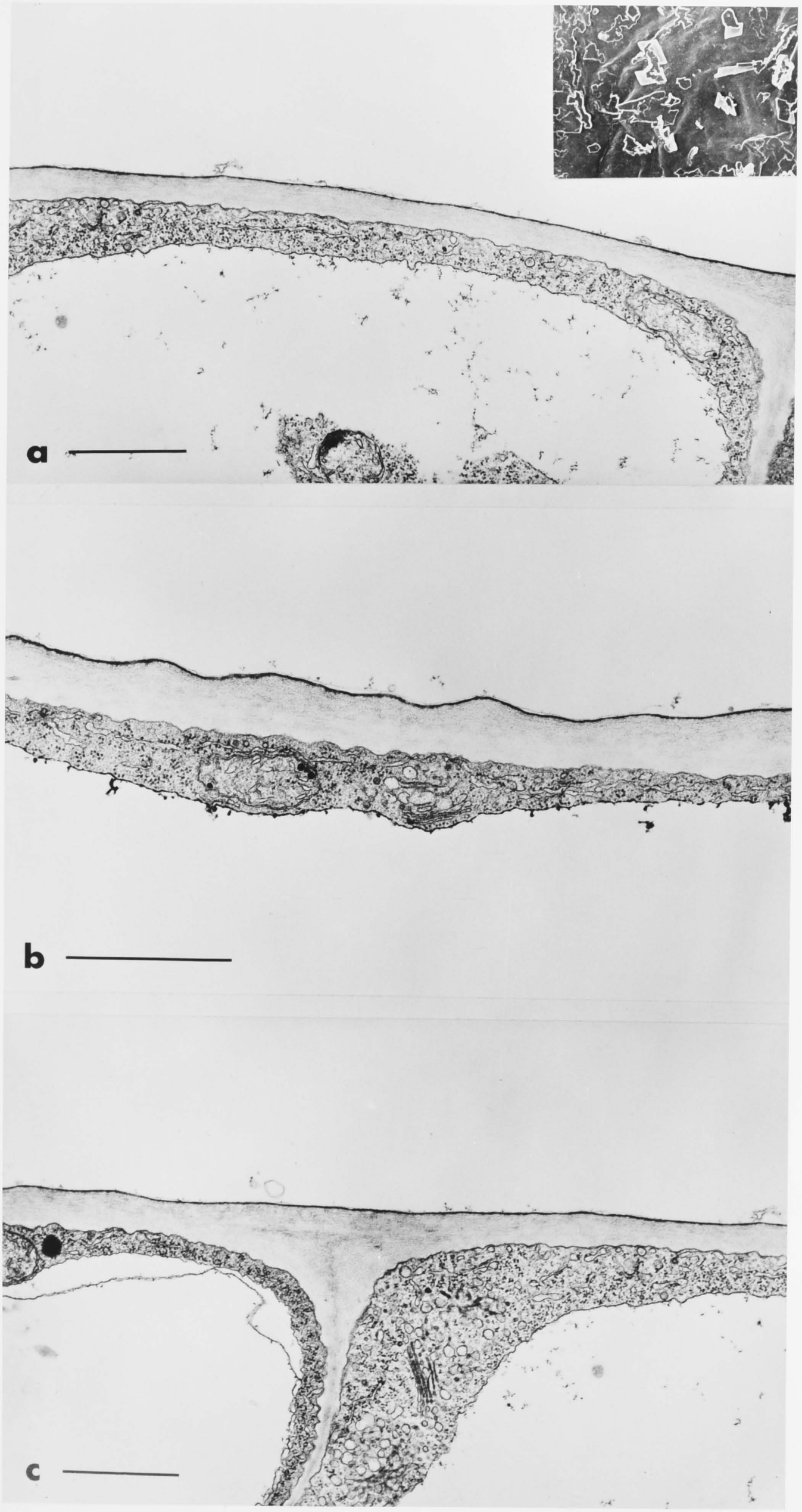
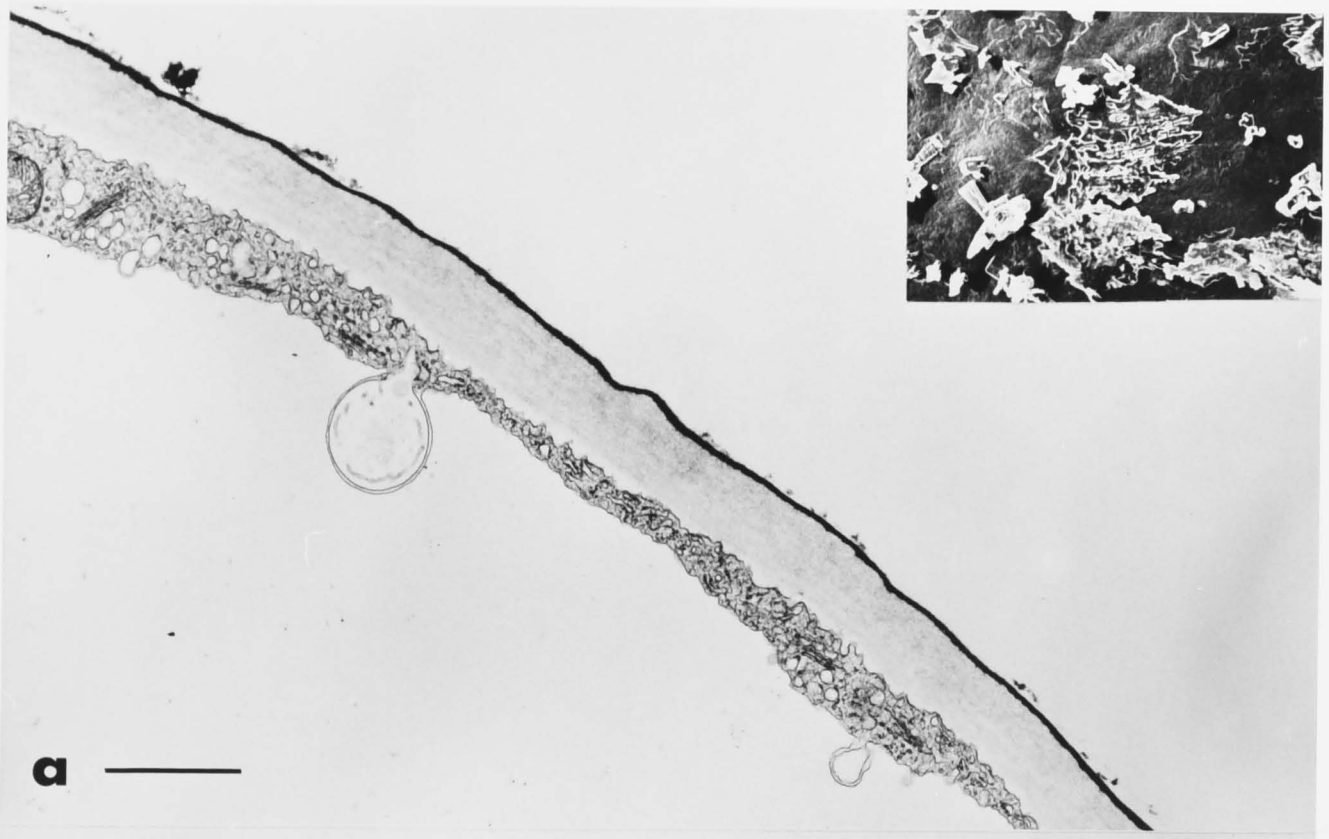
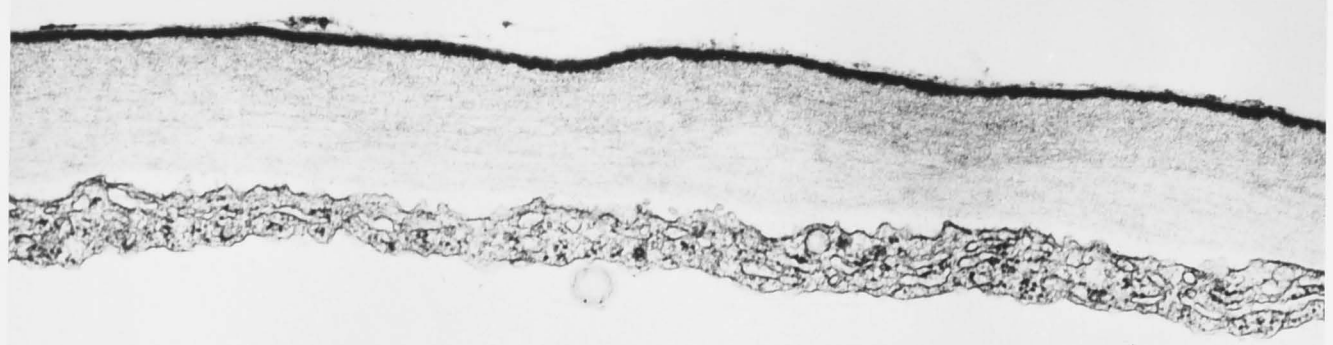

a
b
c

Plate 1.9 a,b,c: Electron micrographs illustrating outer epidermal cell wall fine structure of 24/19 °C grown leaves after root applications of T.C.A. (trichloroacetic acid). Inset illustrates surface wax features.


a


b

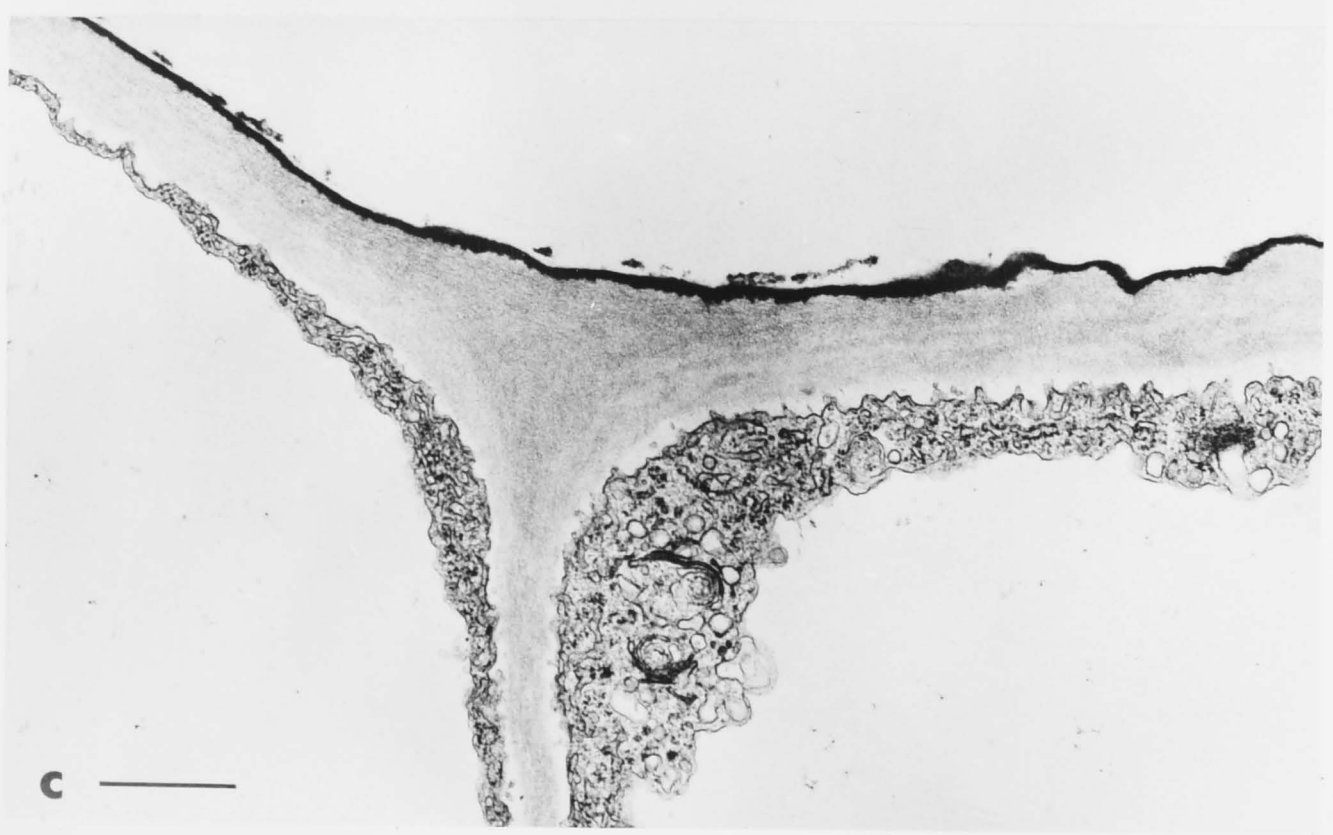
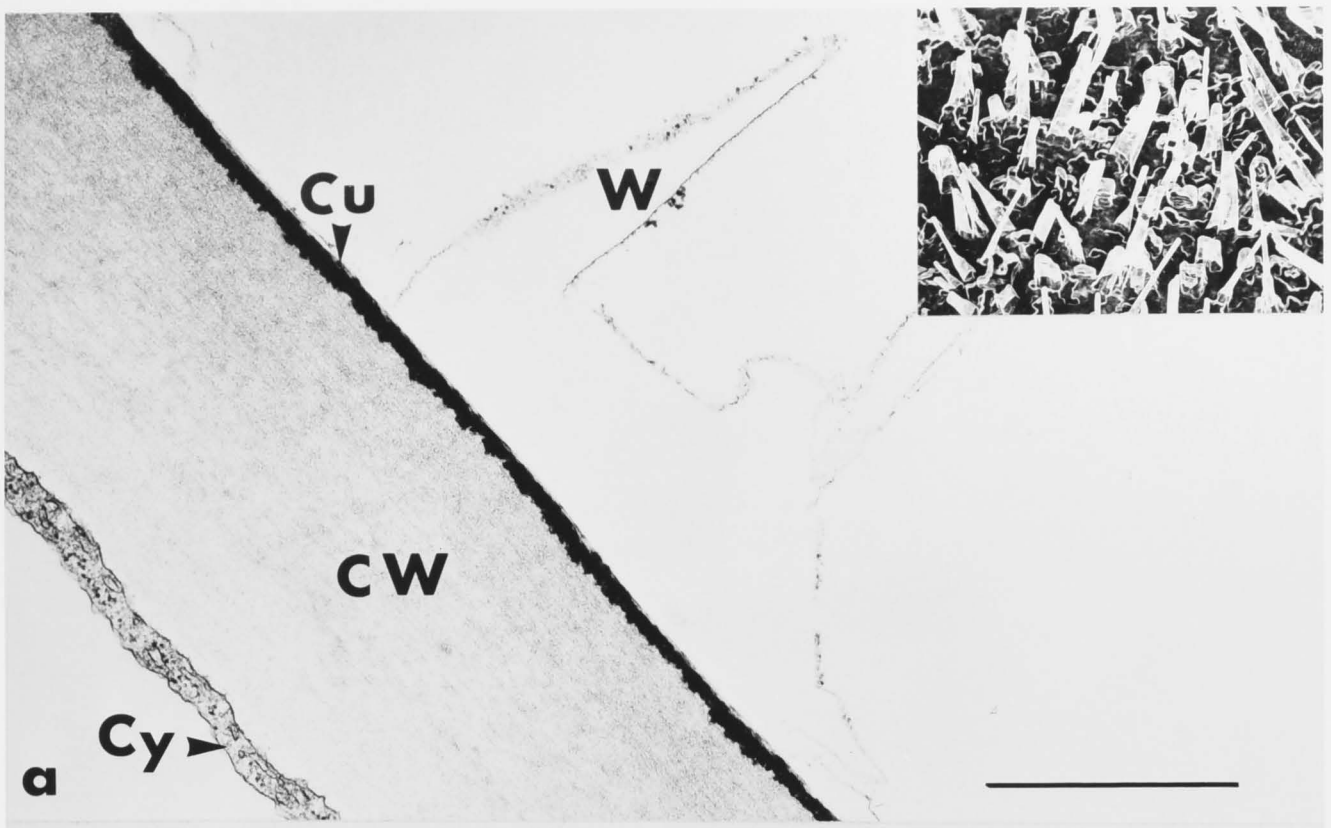

c

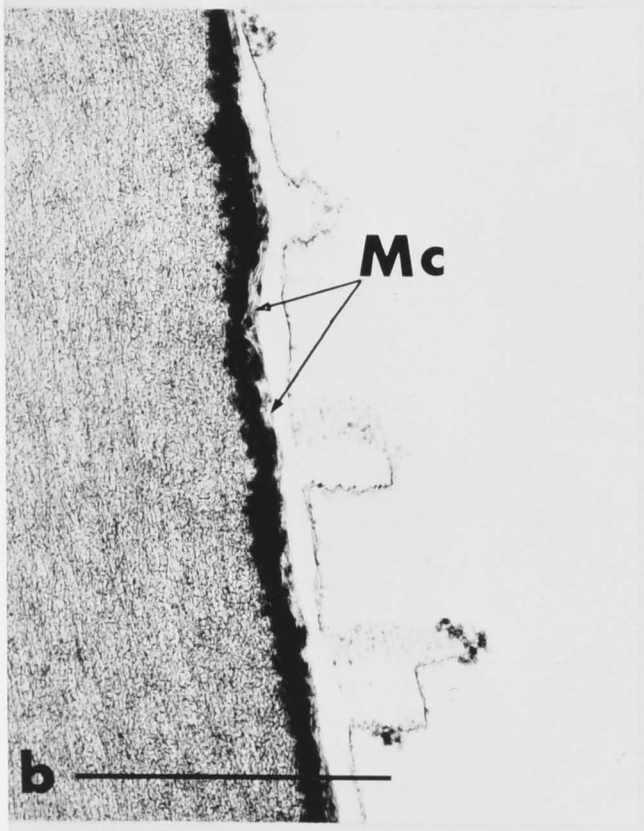
Plate 1.10: Electron micrographs illustrating outer epidermal cell wall features of a 15/10 °C grown plant.

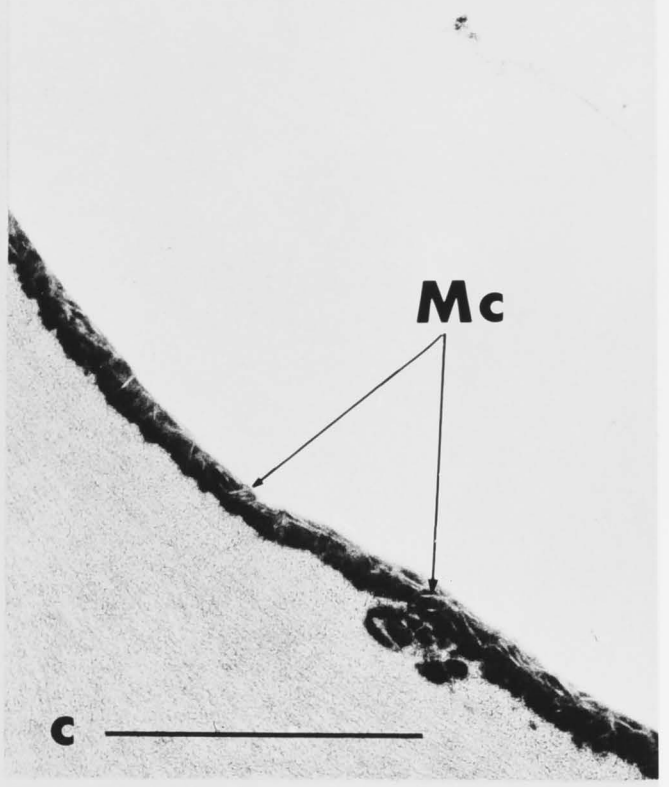
(a) Section illustrates the cuticle (Cu) with its associated surface wax (W), cell wall (CW) and cytoplasm (Cy). Inset illustrates surface wax characteristics.

(b,c) Cuticle-cell wall region showing microchannels (Mc) ~ 7 nm in diameter.

(d) Higher magnification detail of the cuticle showing microchannels which traverse the entire width.


Cu
W
CW
Cy
a


Mc
b


Mc
c

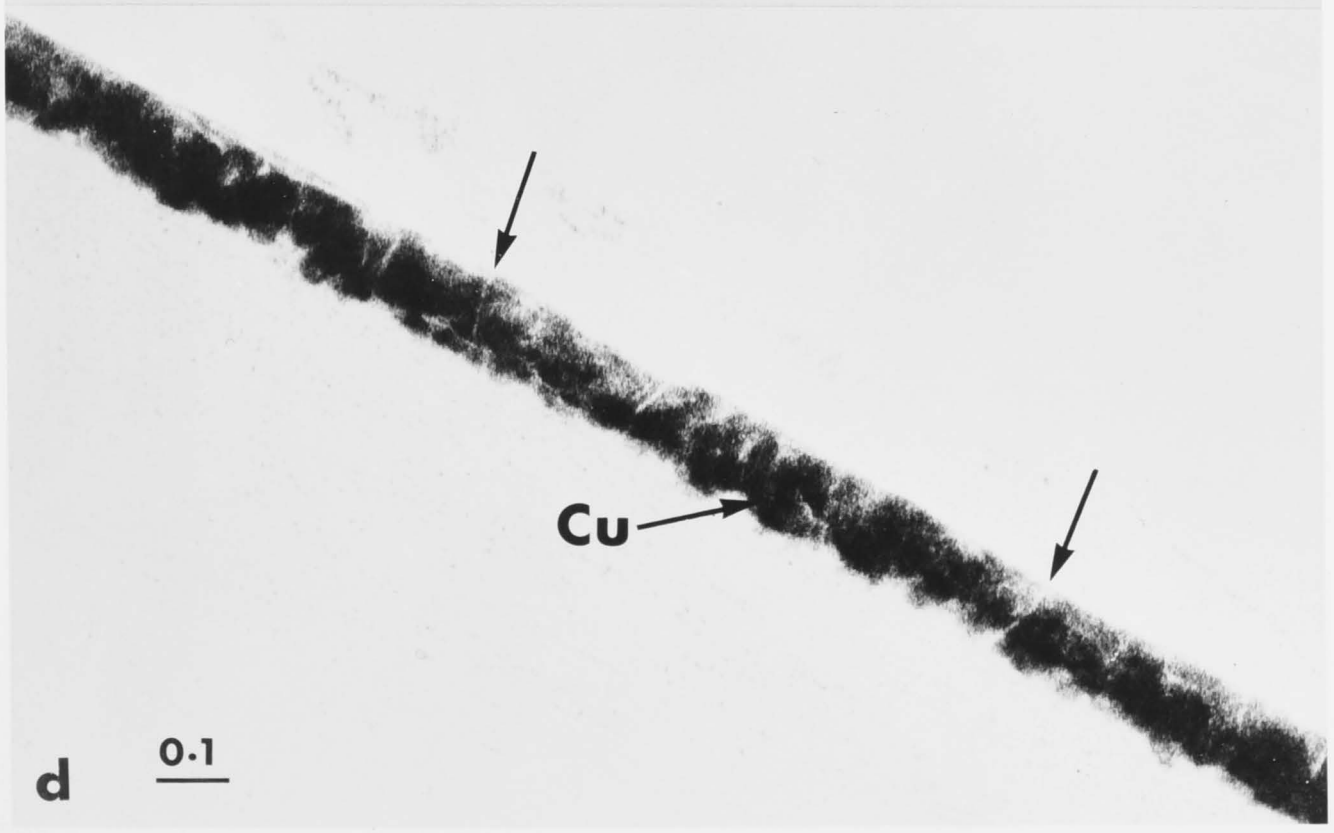
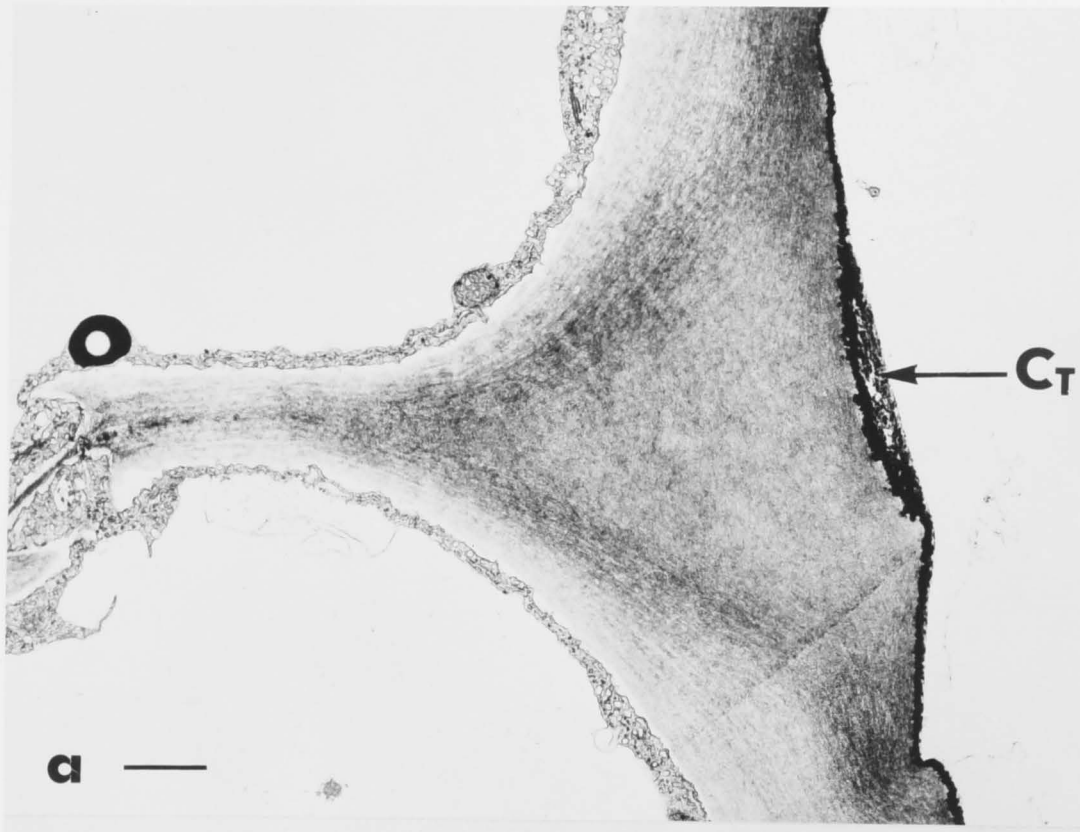

Cu
d
0.1

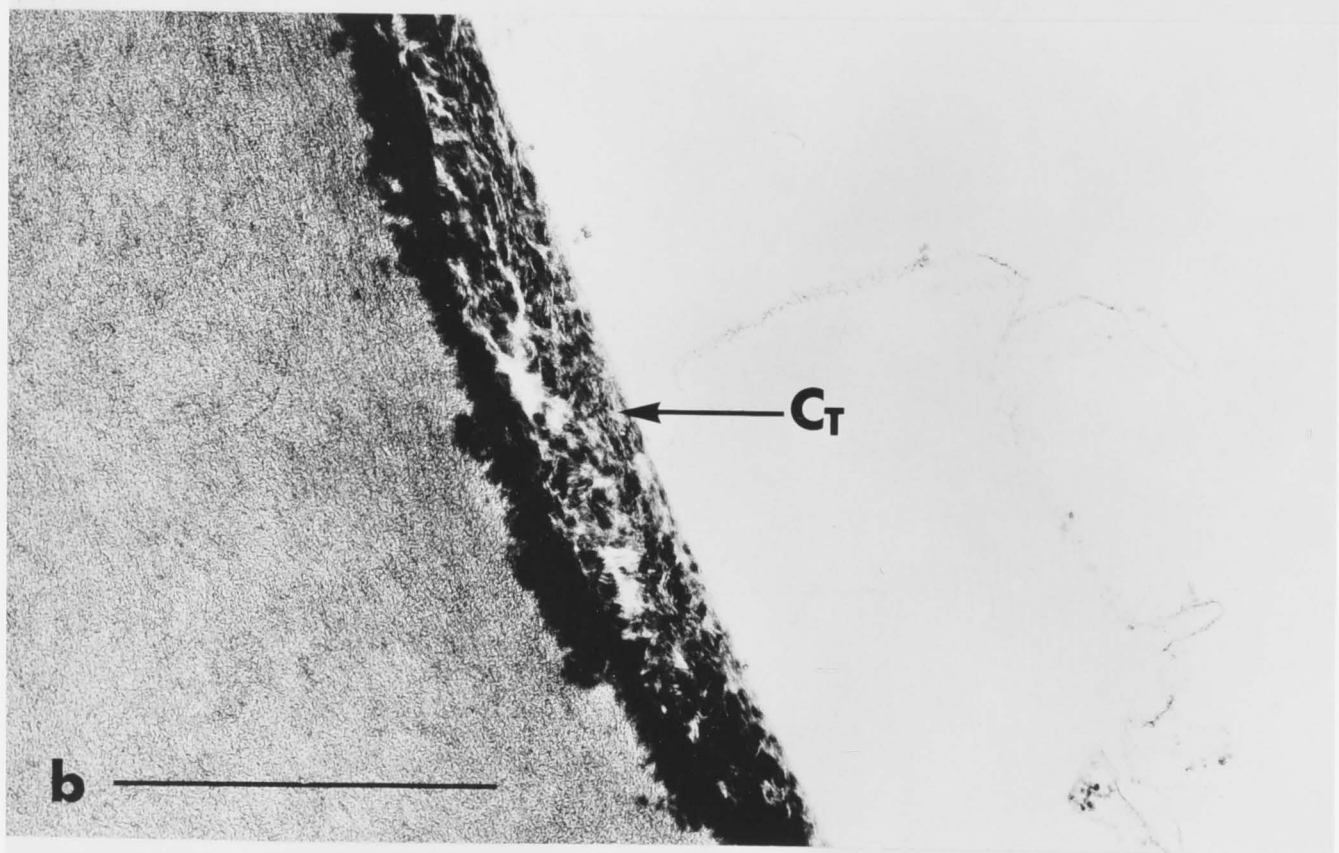
Plate 1.11: Electron micrographs illustrating an outer epidermal cell wall junction for a 15/10 °C grown plant exhibiting:

(a) a cuticular thickening (C_T);

(b) the same area C_T in Plate a at higher magnification;

(c) a cuticular thickening when sectioned parallel to the leaf surface with low electron dense strand regions (M_E) and densely stained pore-like areas (P) ~ 7 nm in diameter.


O
C_T
a


C_T
b

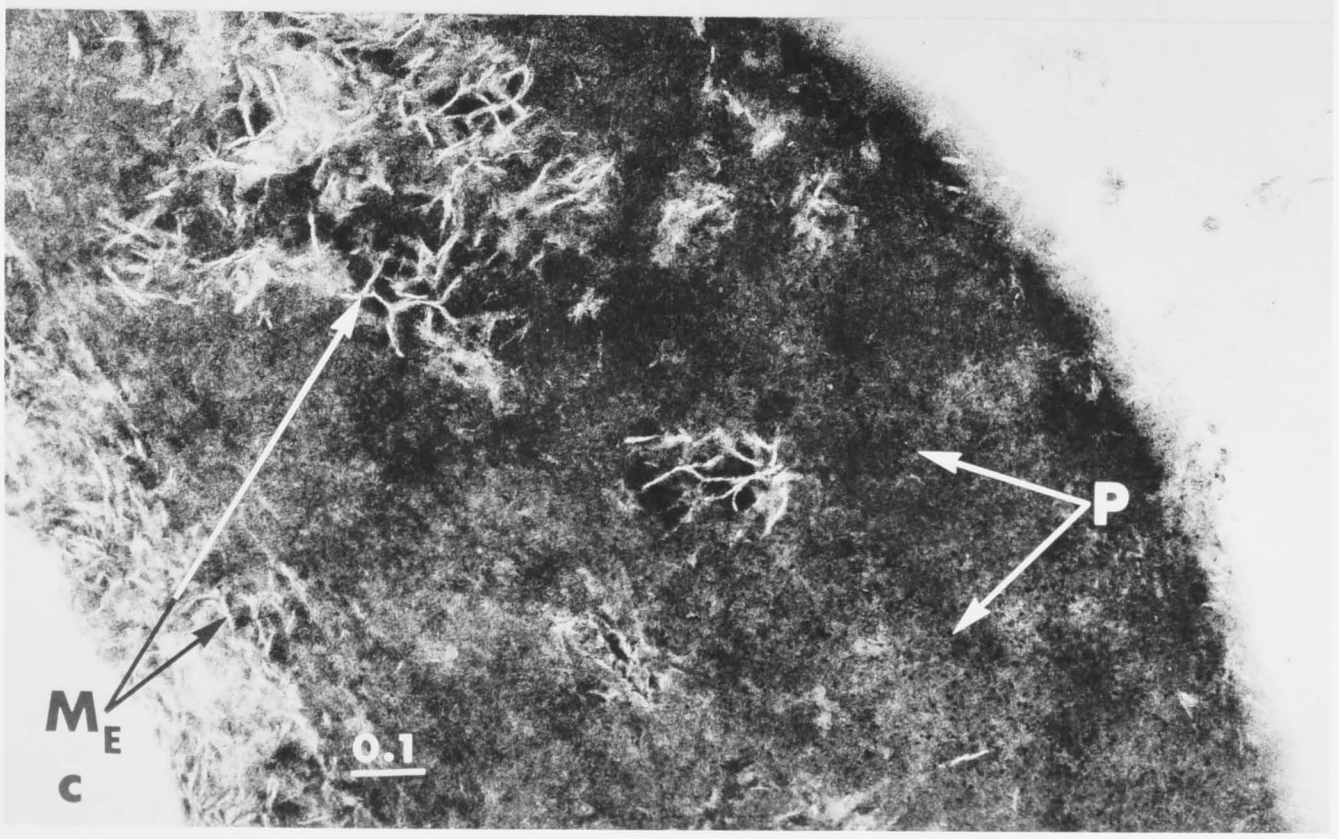
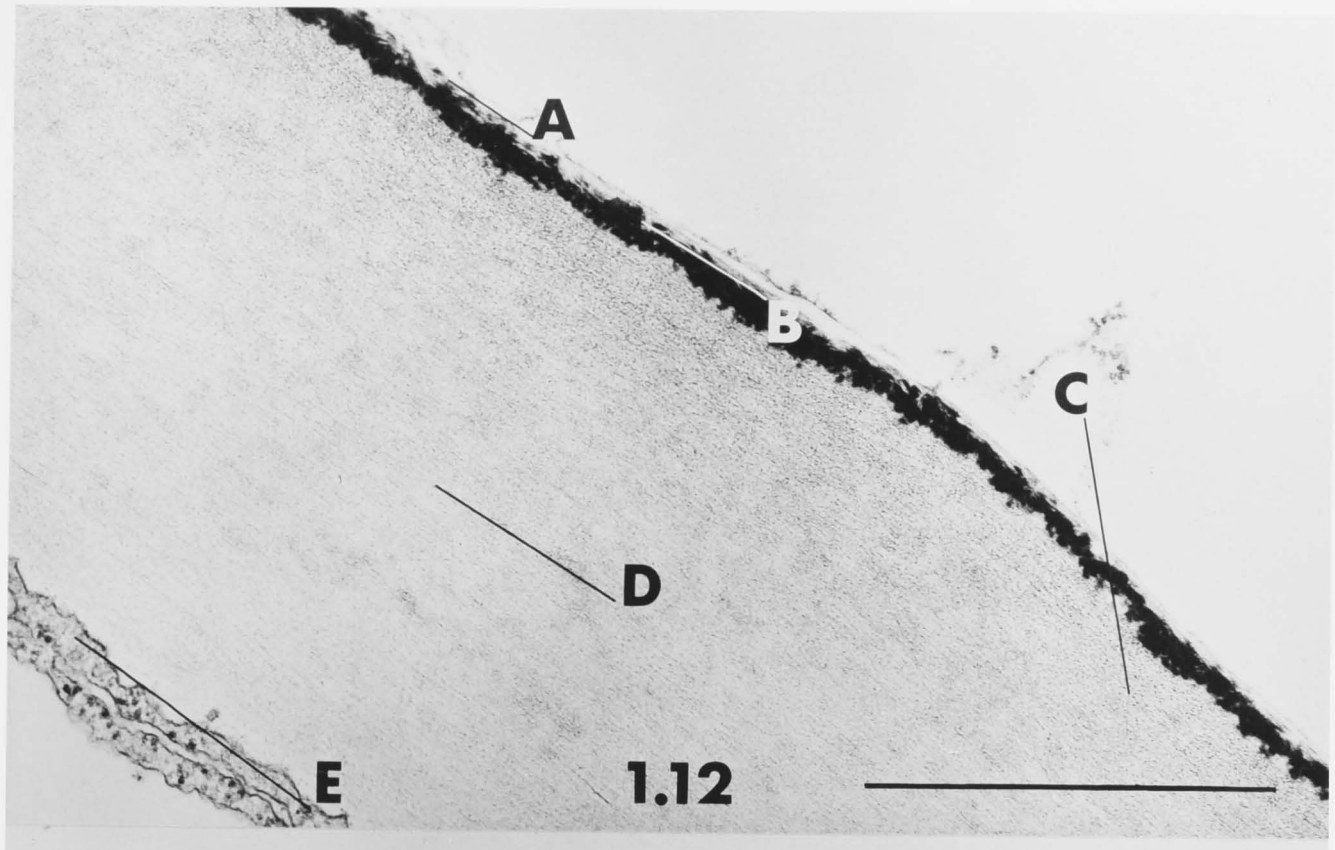

P
M_E
0.1
c

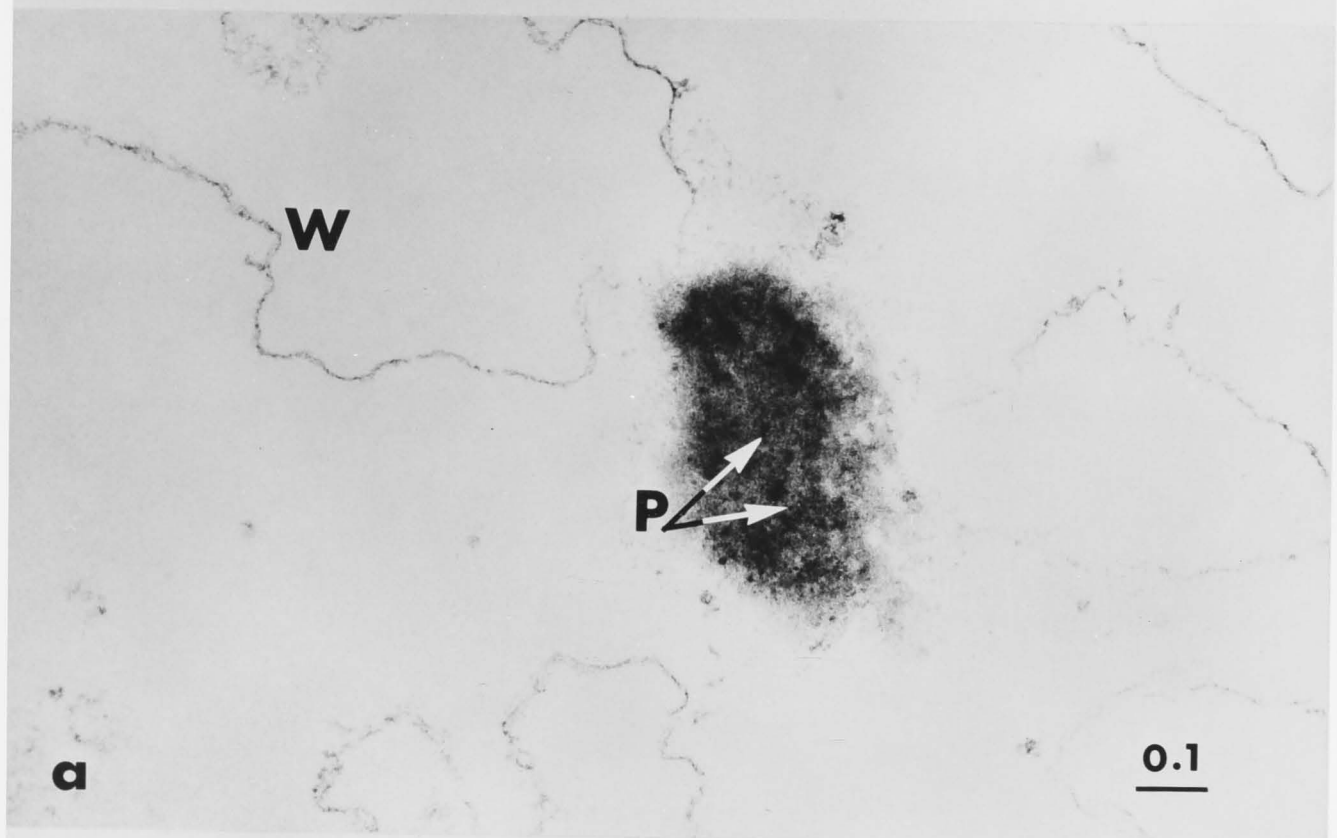
Plate 1.12: Electron micrograph illustrating the surface wax, cuticle, cell wall and cytoplasm of an epidermal cell of a 15/10 °C grown plant.

Section lines A - E indicate the level at which sections were cut parallel to the leaf surface as illustrated in Plates 1.12 a - e below and overleaf.

Plate 1.12a: Section line A — an outermost region of the cuticle showing 'pore'-like areas (P) ~ 7 nm in diameter and sectioned surface wax (W).

Plate 1.12b: Section line B — section cut further into the cuticle, again showing 'pore'-like areas (P) and grouped areas of microchannels (Mc) ~ 7 nm in diameter.


A
B
C
D
E
1.12


W
P
a
0.1

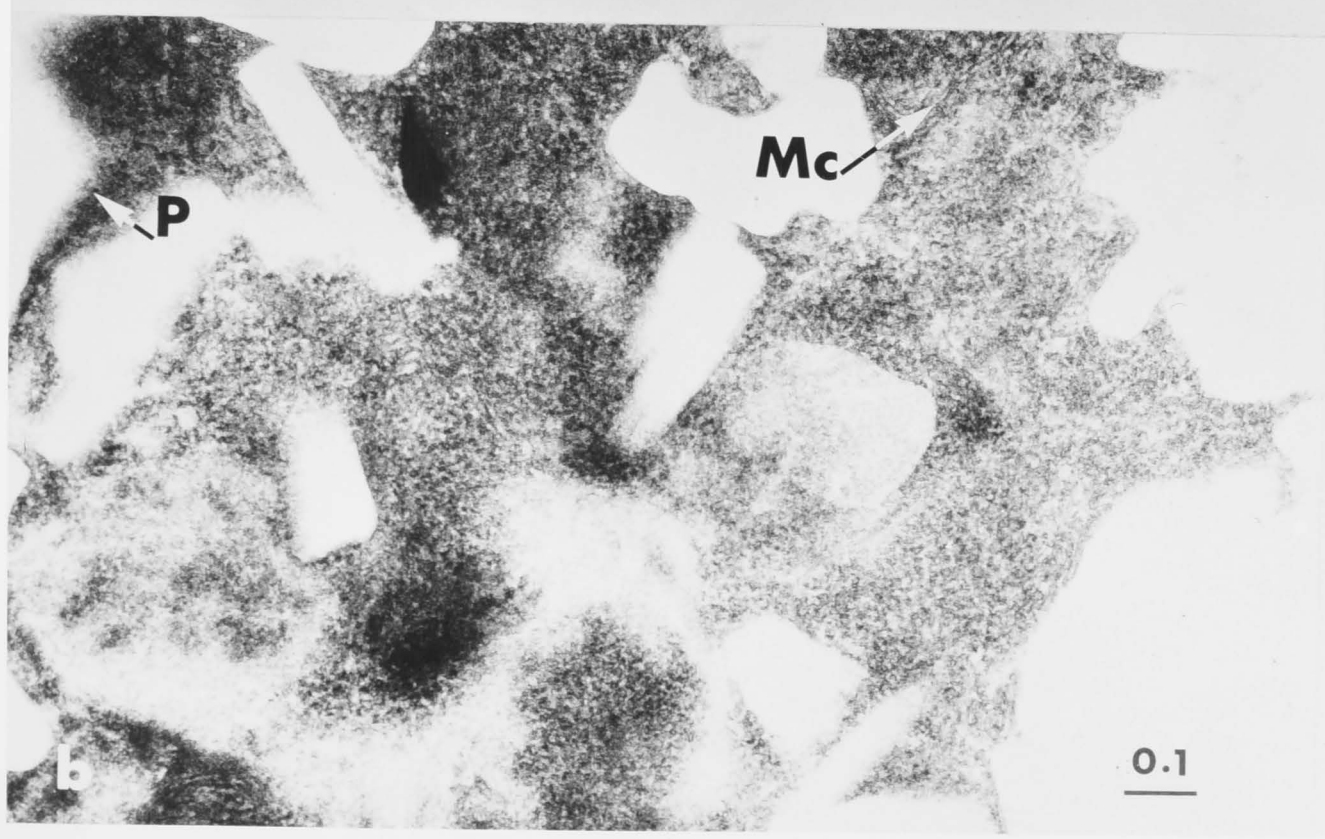

Mc
P
b
0.1

Plate 1.12c: Section line C — section cut obliquely across the leaf surface, illustrating surface wax (W), cuticle (Cu), cell wall (CW), and 'pore'-like areas (P).

Plate 1.12d: Section line D — section cut across the cell wall (CW).

Plate 1.12e: Section line E — section cut across the plasmolemma region showing the bounding cell wall and cytoplasm.

CW — cell wall

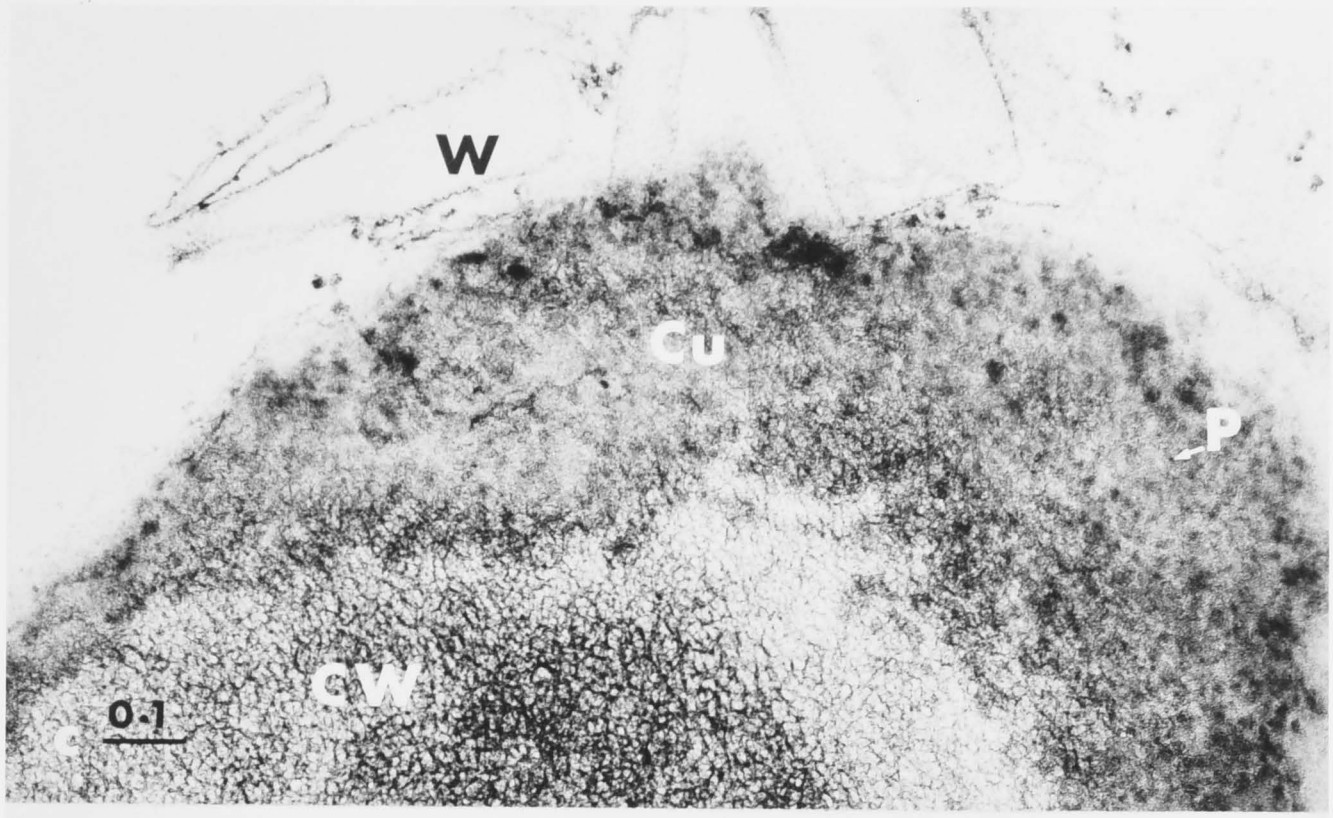
C_N — area of cell wall synthesis

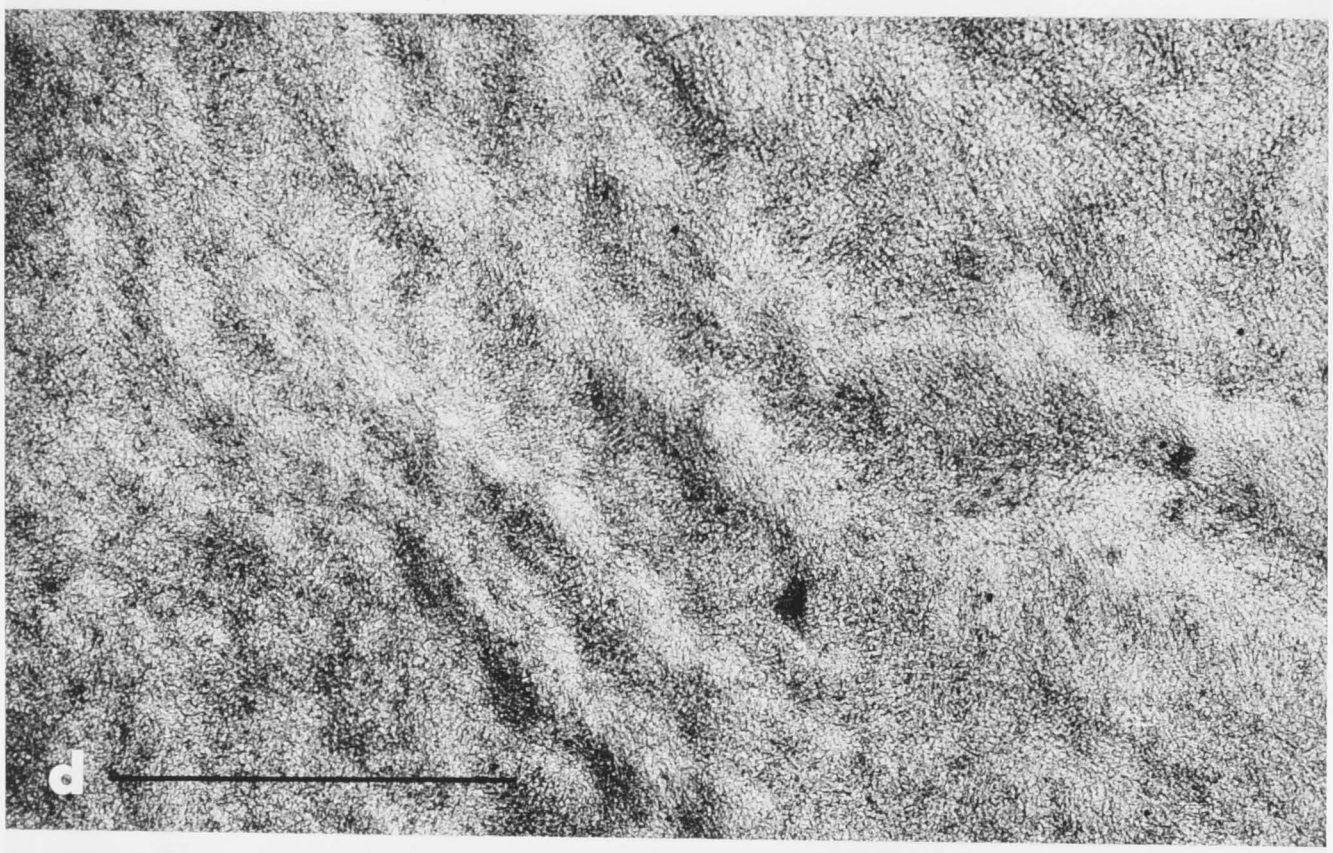
Pl — plasmolemma

Cy — cytoplasm

M — microtubules

R — ribosomes.


W
Cu
P
CW
0.1


d

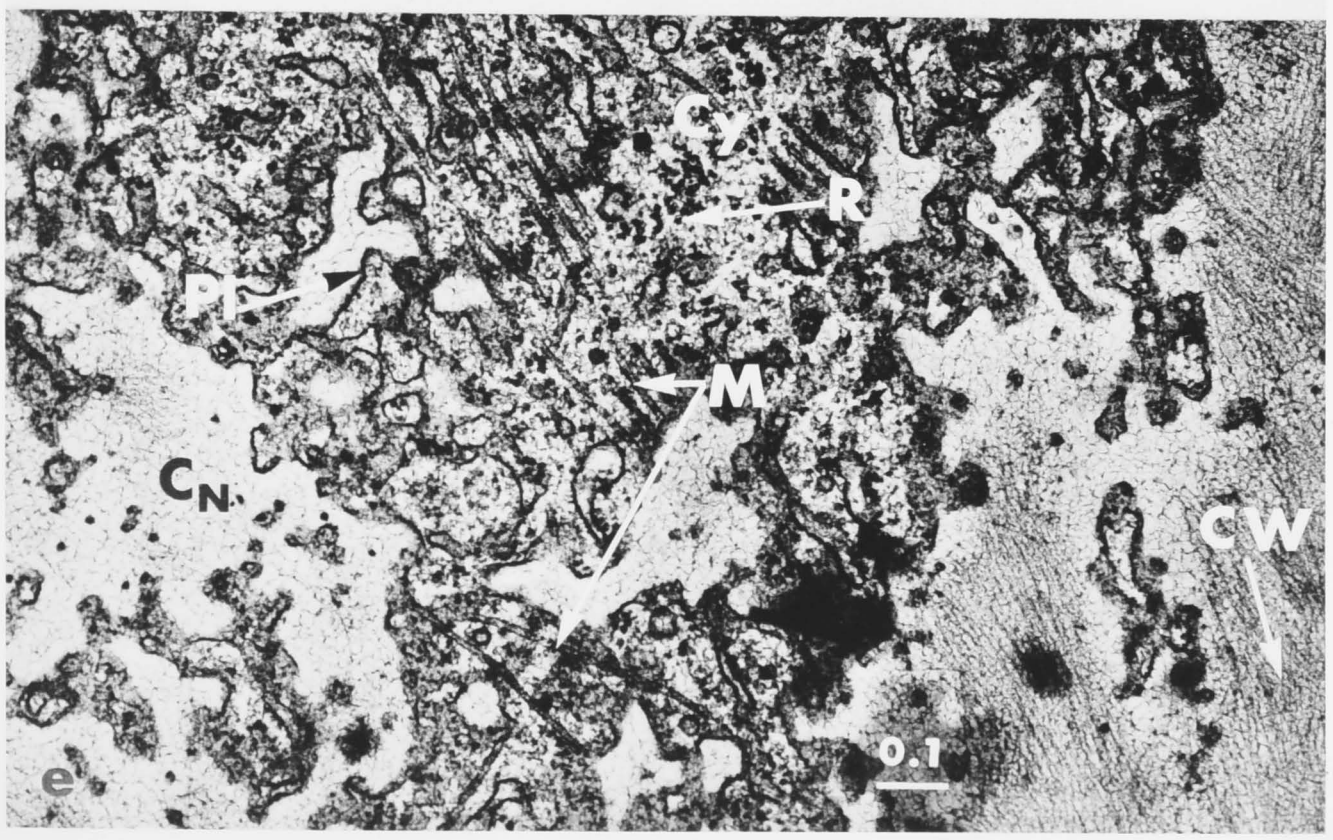
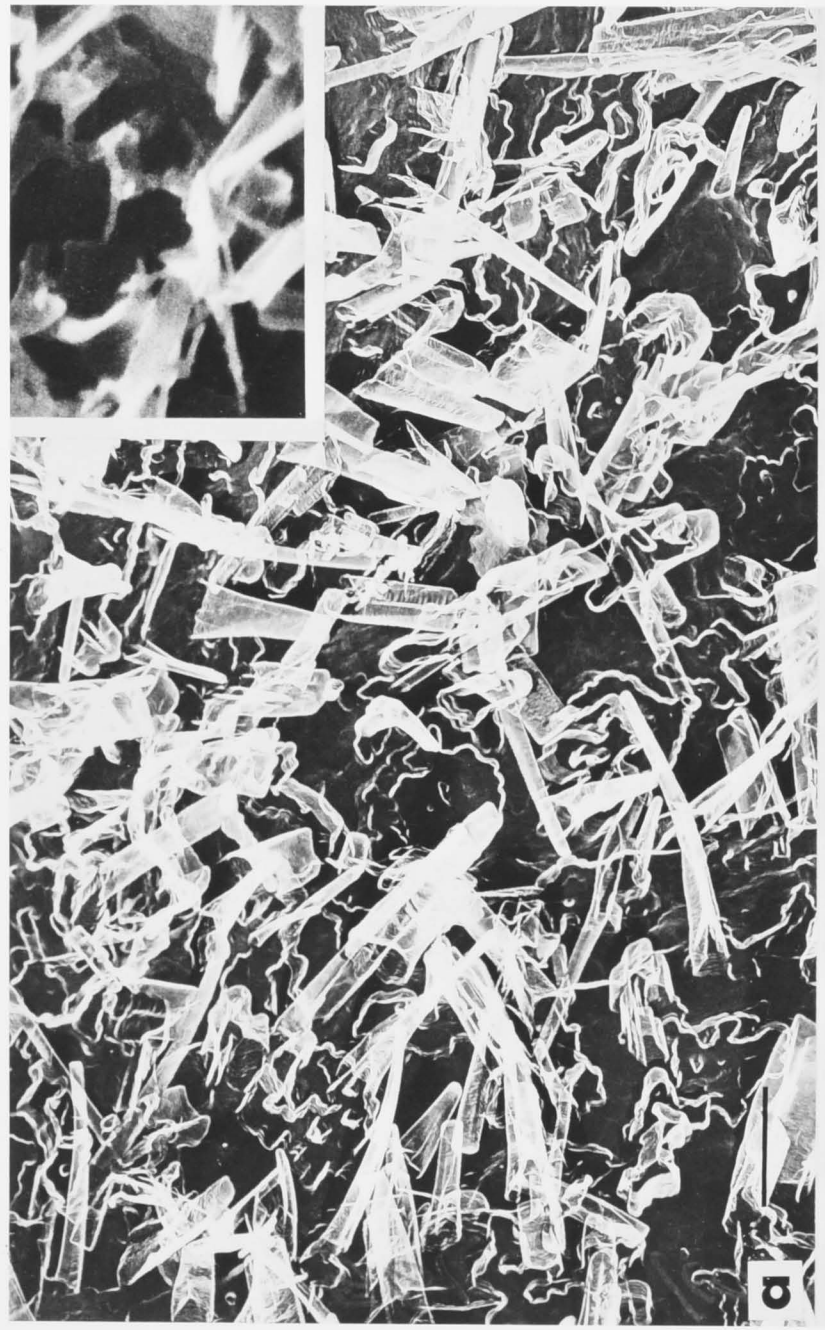

CY
R
PF
M
CN
CW
0.1
e

Plate 1.13: Electron micrographs illustrating leaf surface waxes characteristic of a constant growing temperature regime of

(a) 15/10 °C. Inset illustrates the same wax as viewed with a scanning electron microscope.

(b) 18/13 °C.

(c) 21/16 °C.


a


b

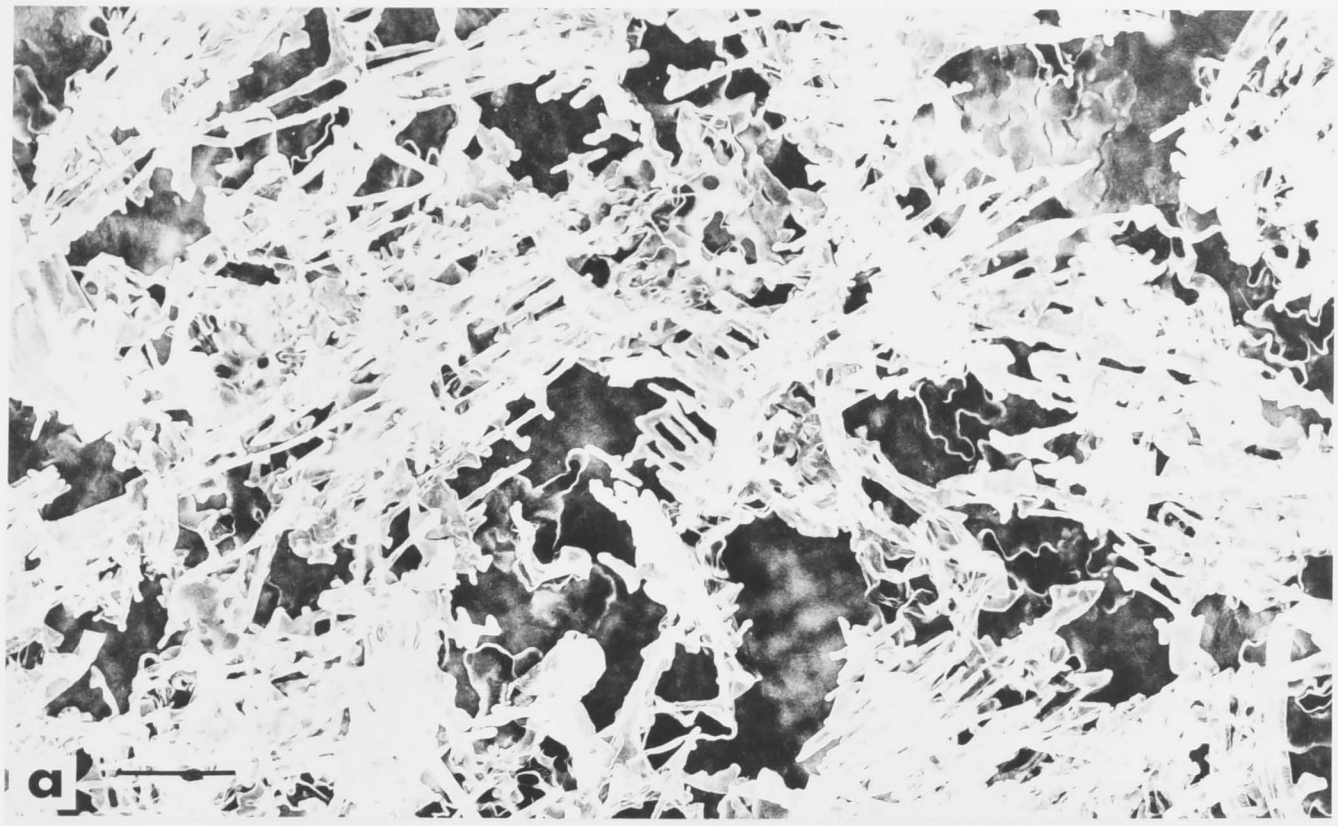

c

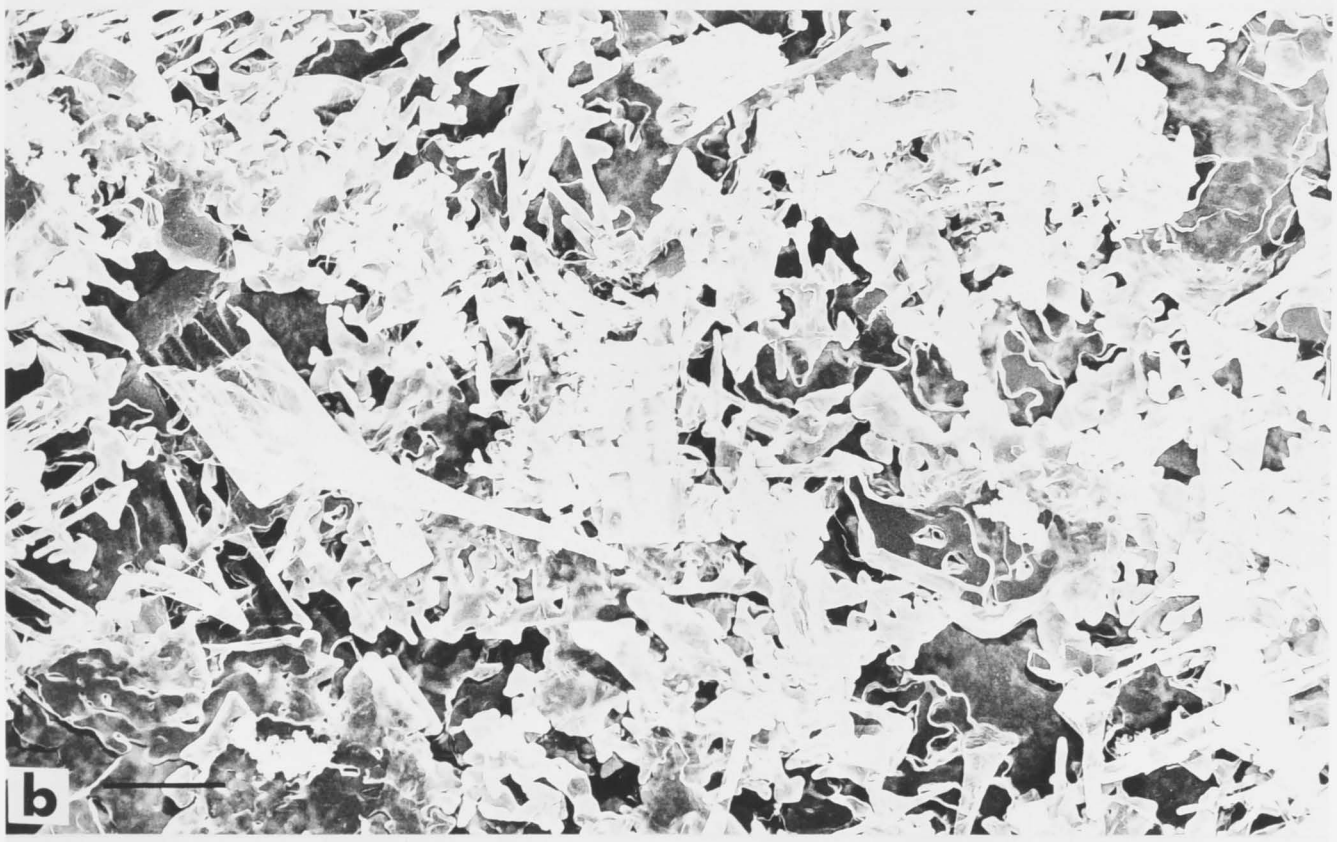
Plate 1.14: Electron micrographs illustrating leaf surface waxes characteristic of a constant growing temperature regime of

(a) 24/19 °C.

(b) 27/22 °C.

(c) 30/25 °C.


a


b


c

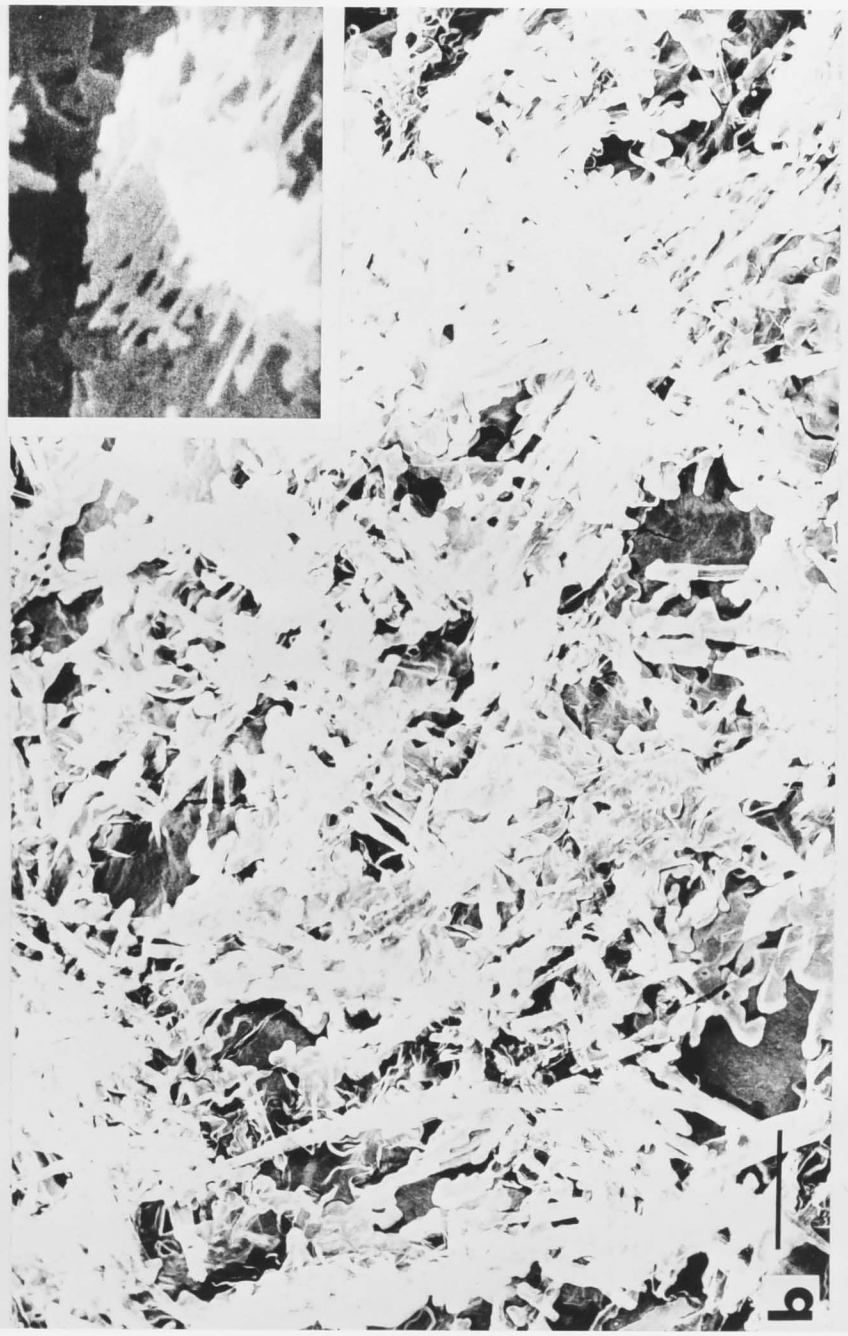
Plate 1.15: Electron micrographs illustrating leaf surface waxes characteristic of constant growing temperature regime of

(a) 33/28 °C.

(b) 36/31 °C. Inset illustrates the same wax as viewed with a scanning electron microscope.

(c) non-waxy (gl_3) mutant.


a


b

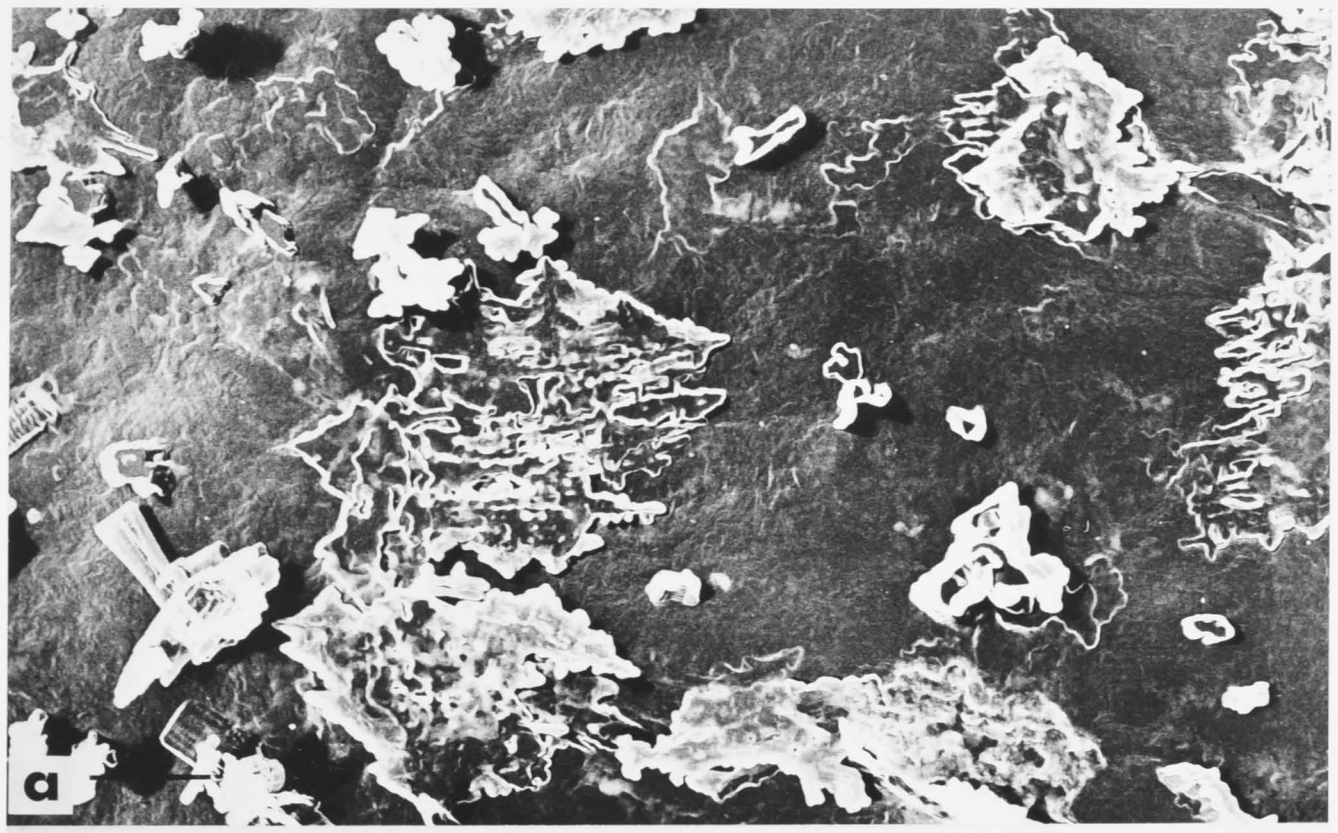

c

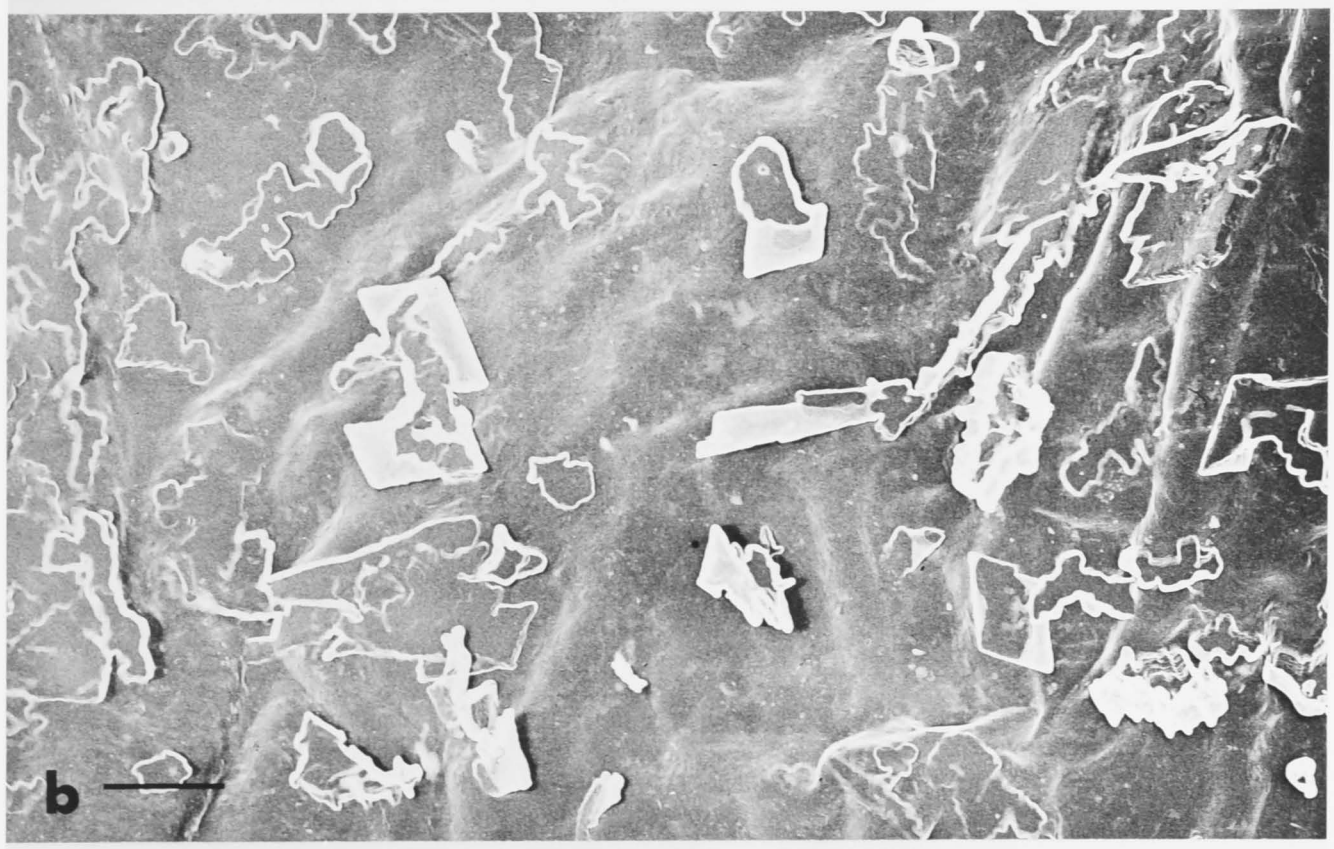
Plate 1.16: Electron micrographs illustrating the leaf surface waxes of 24/19 °C grown plants after root applied herbicide treatments

(a) T.C.A. (trichloroacetic acid).

(b) Dalapon (2,2-dichloropropionic acid).

(c) control.


a


b

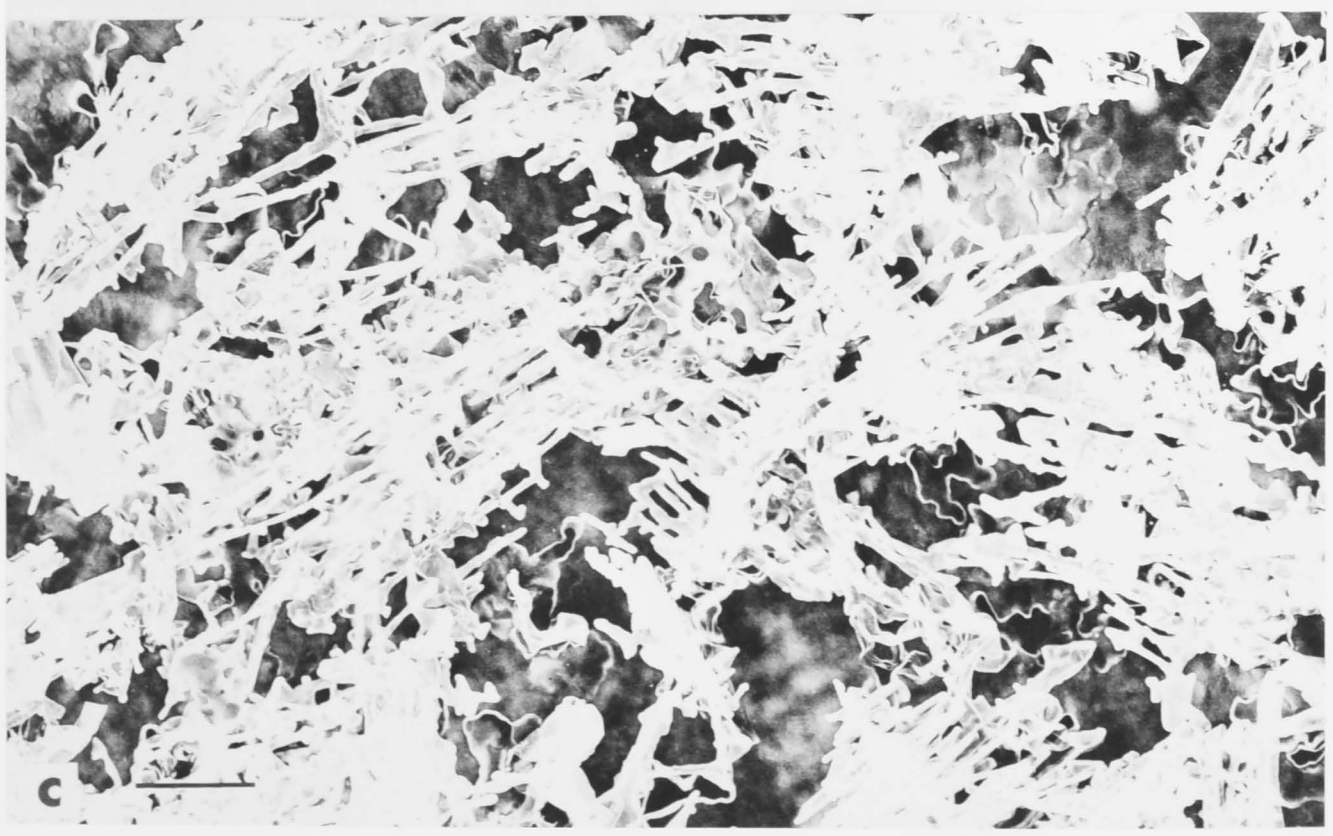
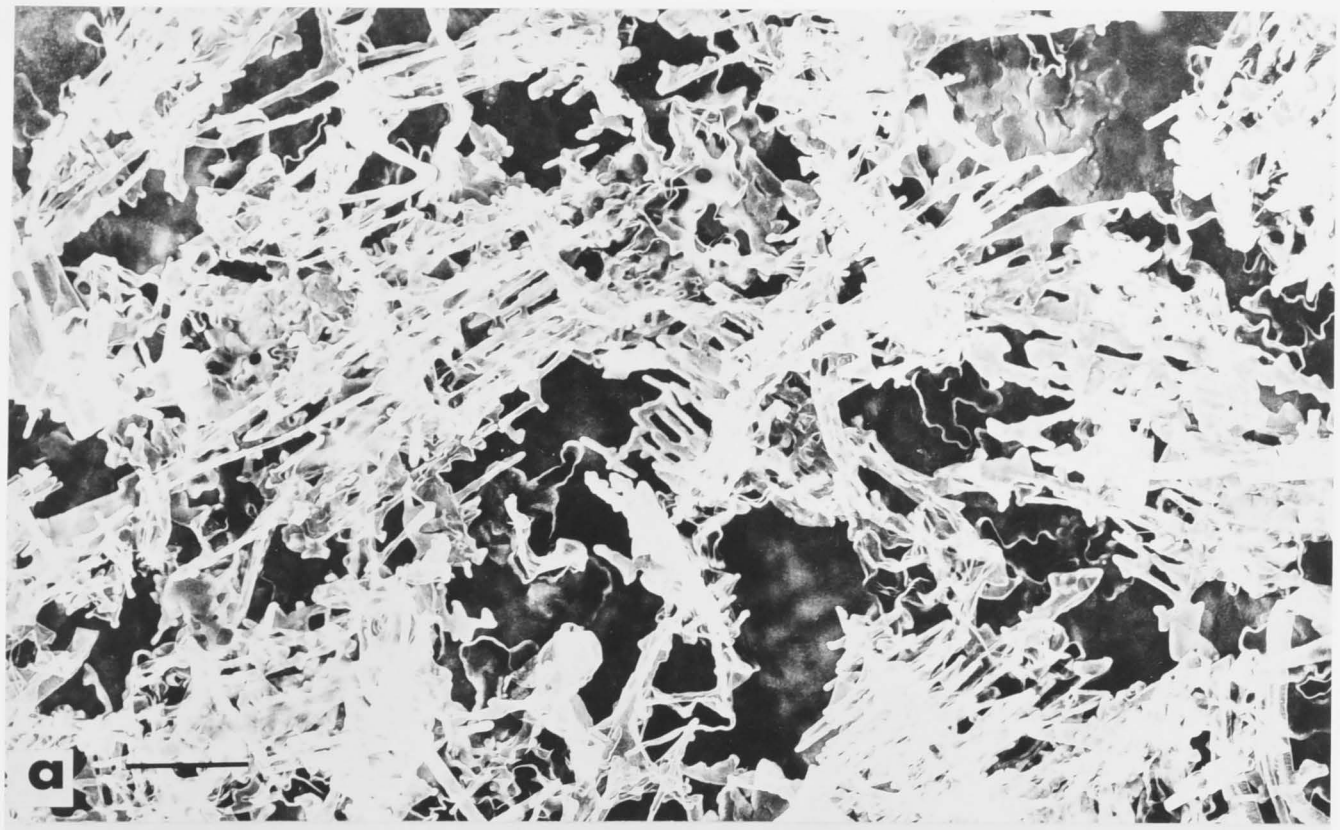

c

Plate 1.17: Electron micrographs illustrating leaf surface wax and its modification by treating 24/19 °C grown plants with two root applications of T.C.A.

(a) Surface prior to treatment.

(b) Surface of the same leaf as (a) after treatment.

(c) Surface after treatment of a fully-expanded leaf.


a


b

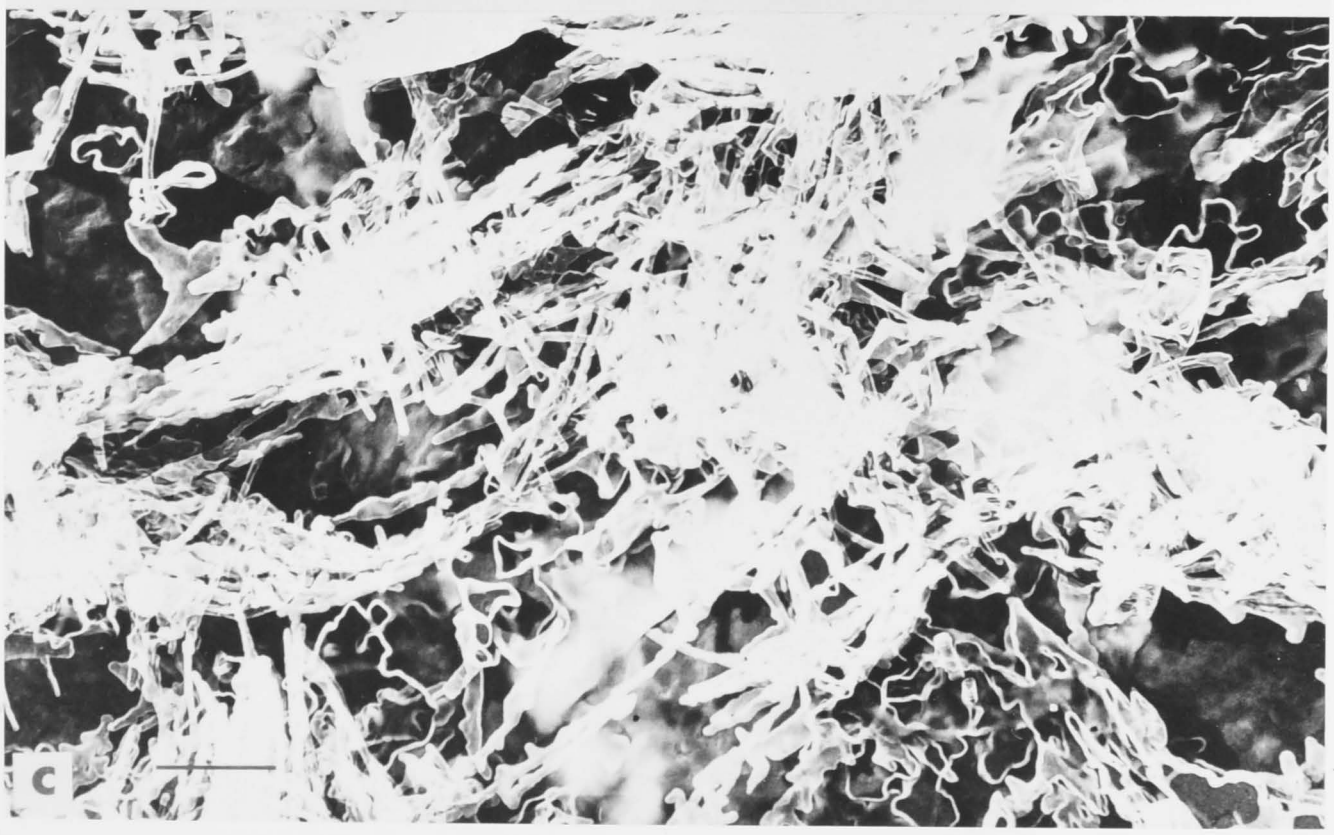

c

Plate 1.18: Electron micrographs illustrating the typical leaf surface wax pattern for plants grown at

(a) 15/10 °C

(b) 36/31 °C.

These controls are included for comparison with Plates 1.19 a,b,c; 1.20 a,b,c; 1.21 a,b,c, which illustrate the modification of low temperature wax resulting from transfer to higher temperature conditions.


a


b

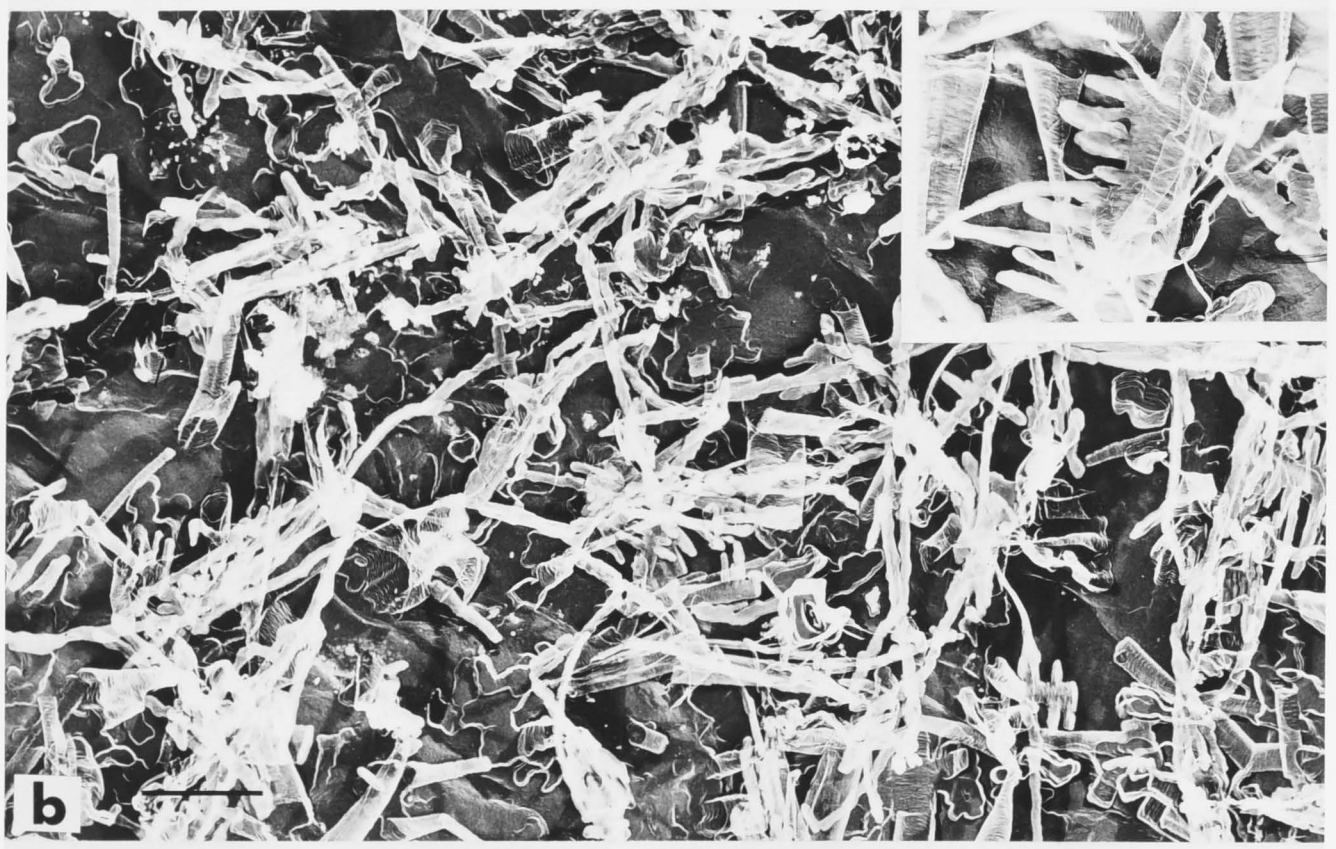
Plate 1.19: Electron micrographs illustrating leaf surface wax modifications of a 15/10 °C established plant after direct transfer to a 36/31 °C temperature regime for

(a) 6 hours.

(b) 24 hours. Inset illustrates the typical modification of pre-existing wax.

(c) 48 hours.


a


b

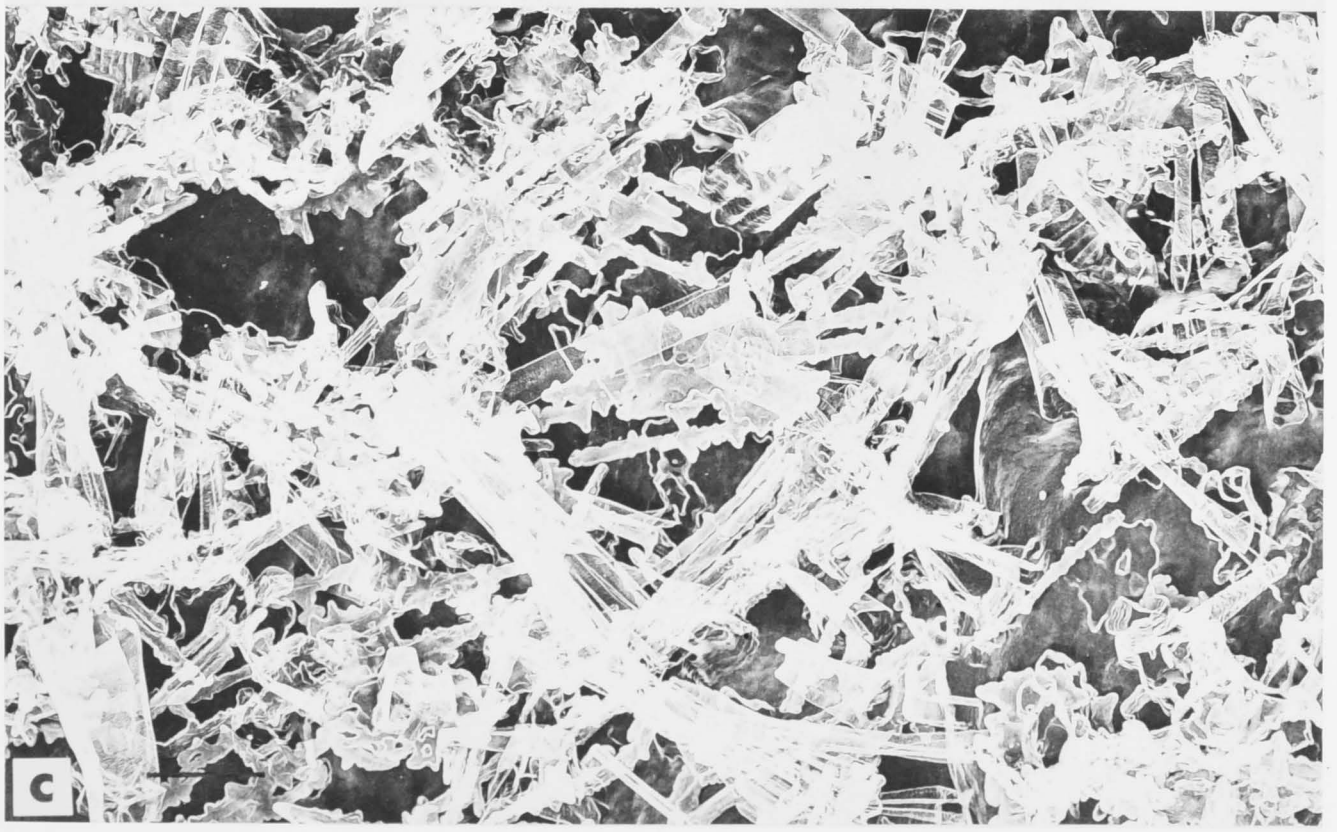

c

Plate 1.20: Electron micrographs illustrating leaf surface wax modifications of a 15/10 °C established plant after direct transfer to a 36/31 °C temperature regime for

(a) 72 hours.

(b) 96 hours.

(c) 120 hours.

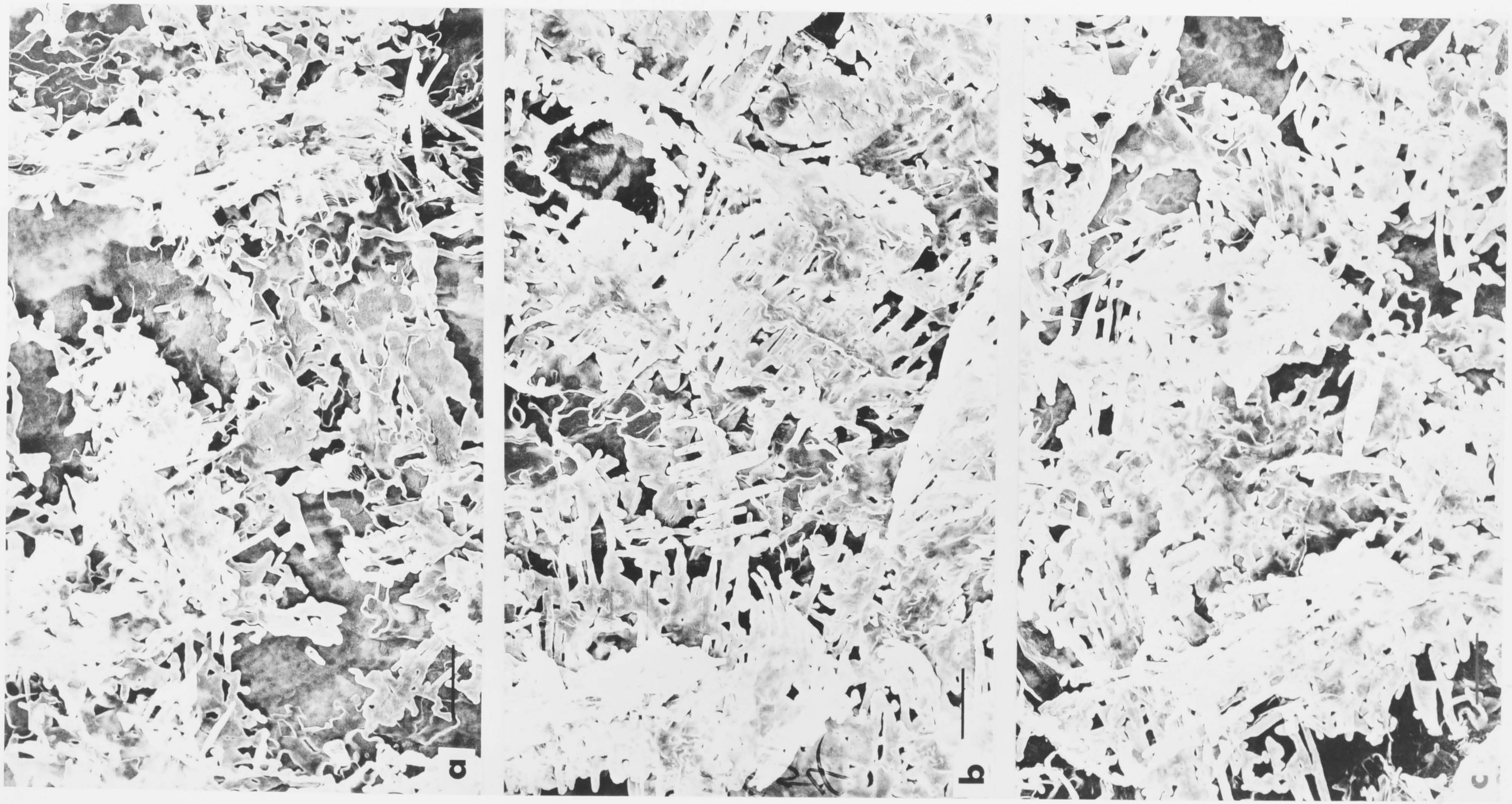

a
b
c

Plate 1.21: Electron micrographs illustrating the leaf surface wax modifications of a 15/10 °C established plant after being transferred to

(a) 36/31 °C for 42 hours (programmed temperature increase 0.5 °C/hour).

(b) 27/22 °C for 24 hours (direct transfer).

(c) 27/22 °C for 24 hours (programmed temperature increase 0.5 °C/hour).

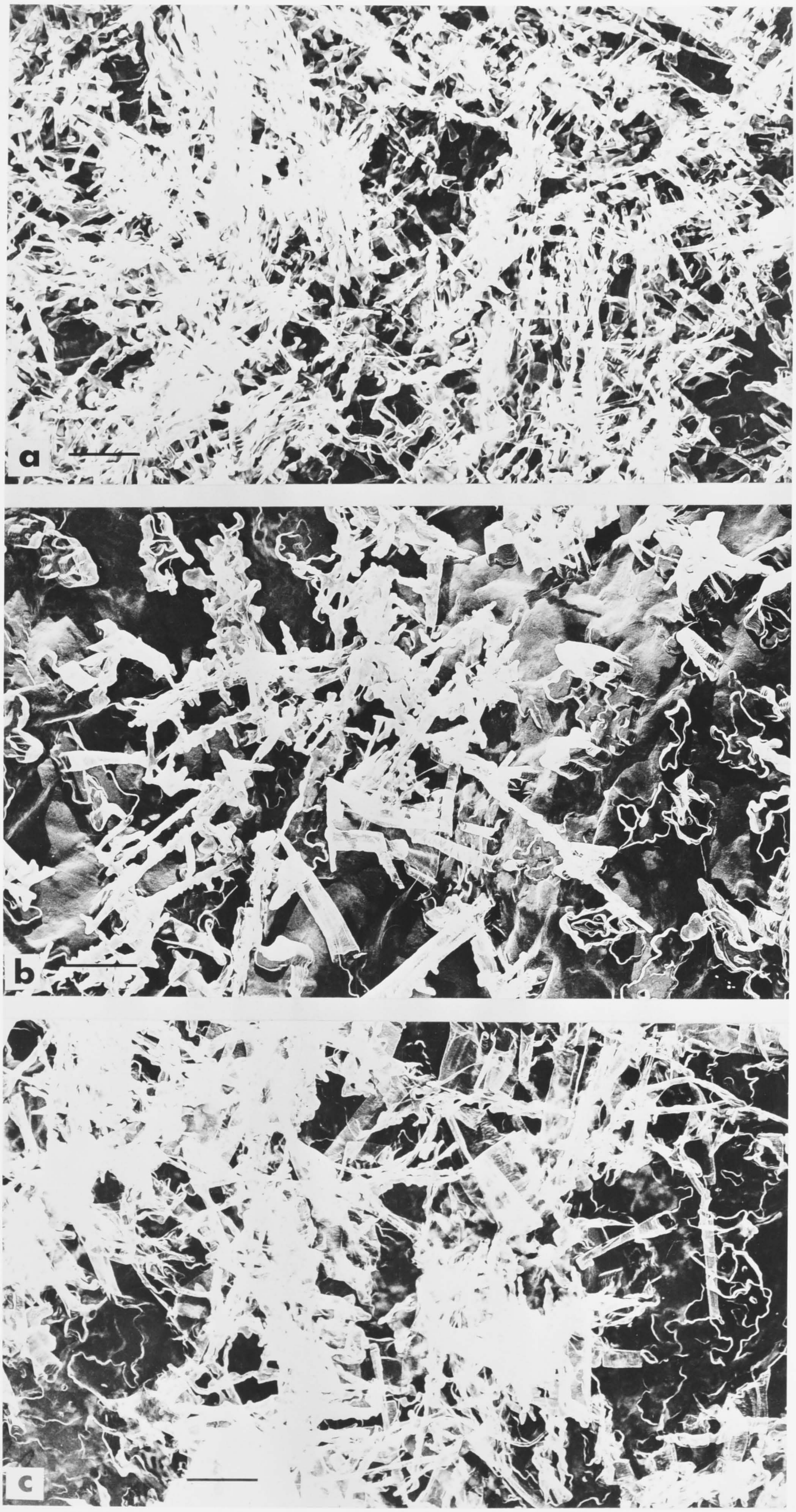

a
b
c

Plate 1.22: (a,b) Electron micrographs illustrating the leaf surface wax modifications of a 15/10 °C grown plant as seen in thin section after direct transfer to 27/22 °C for 24 hours.

(c) illustrates the typical non-branching wax rods characteristic of a 15/10 °C control.

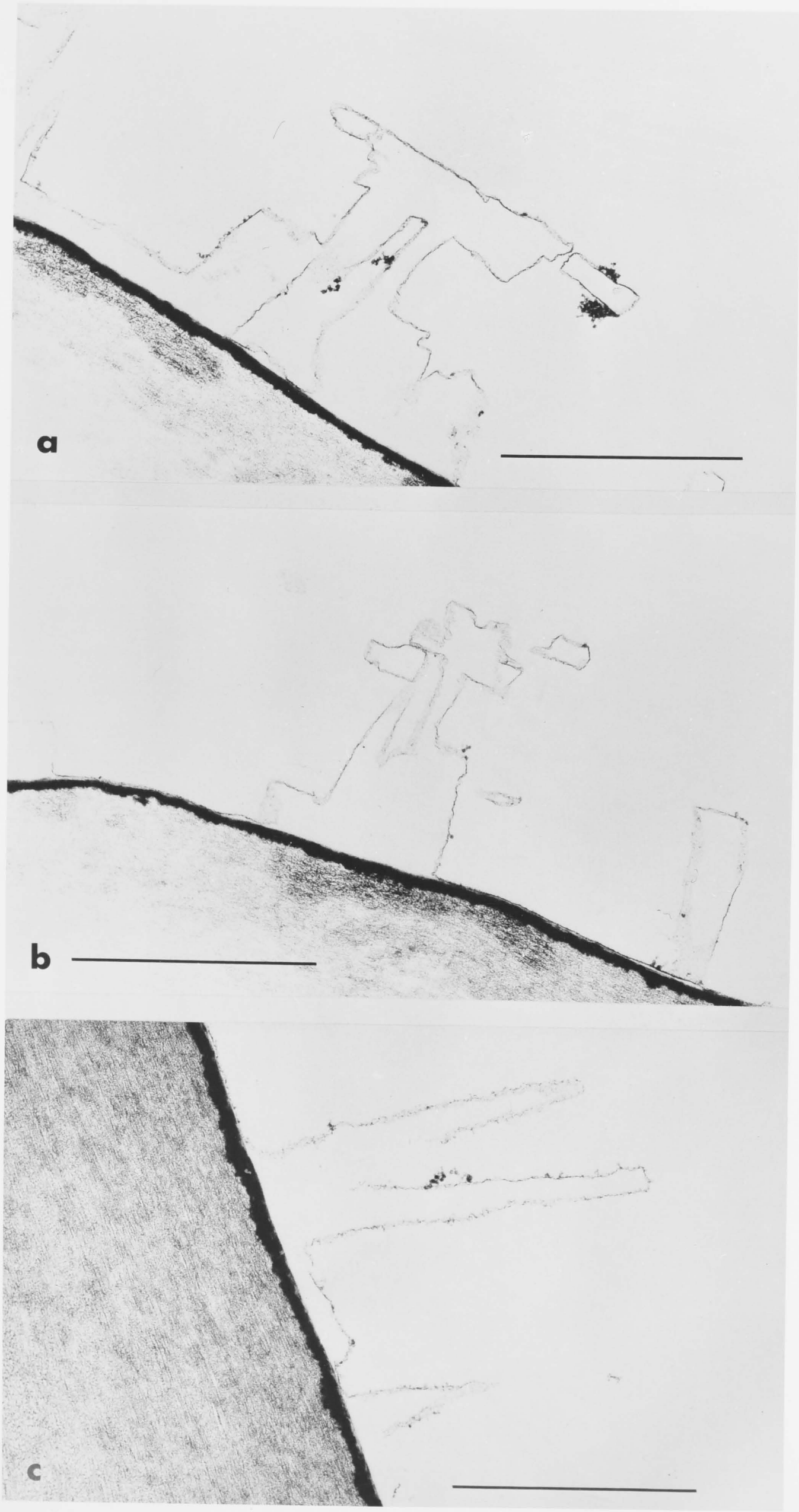
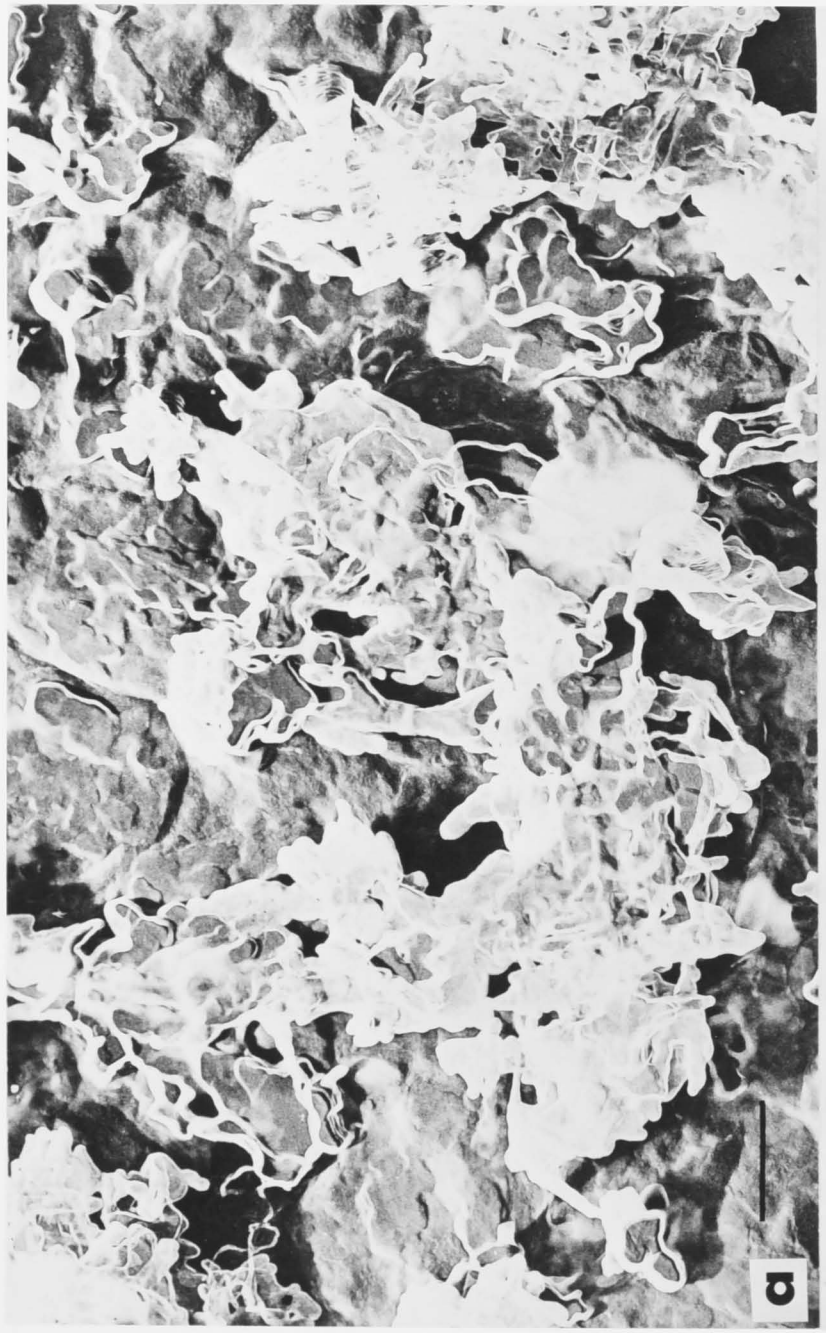

a
b
c

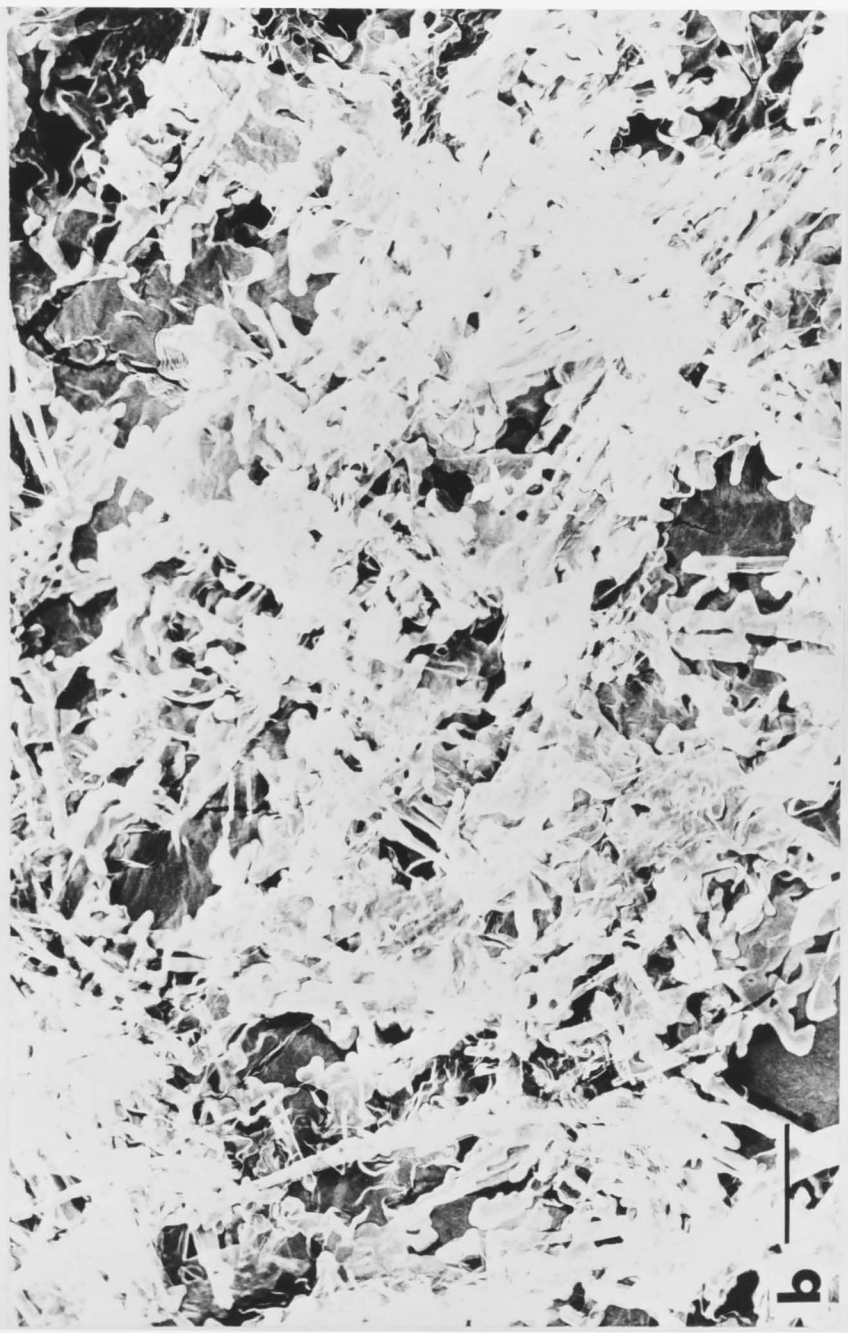
Plate 1.23: Electron micrographs illustrating the leaf surface wax of

(a) a 36/31 °C established plant after direct transfer to 15/10 °C for 120 hours.

(b) a 36/31 °C control.

(c) a 15/10 °C control.


a


b


c

CHAPTER 2

CHAPTER 2

ELECTRON MICROSCOPIC INVESTIGATIONS ON CUTICULAR PORES AND MICROCHANNELS

INTRODUCTION

The transportation of waxes, or their precursors, from their synthetic sites to the cuticular surface has been the subject of much speculation (Crafts & Foy, 1962; Linskens *et al.*, 1965). The involvement of 'pores' or 'channels' was suggested as a result of earlier studies (Dous, 1927; Kreger, 1948; Mueller *et al.*, 1954; Scott *et al.*, 1958; Juniper, 1959b; Schieferstein & Loomis, 1959) to account for the presence of superficial wax deposits. In spite of general agreement on the necessity for microchannels or some similar pathway, concerted efforts to demonstrate such structures have produced very little conclusive evidence.

Pores have also been studied from the aspect of the cuticular surface. Volz (1952) examined the surface directly while Hall & Donaldson (1962) and Hall (1967a) employed two-stage replicas following substantial removal of surface wax with lipid solvent. In the latter studies, the authors related the positioning of the wax structures with the distribution of the cuticular pores, suggesting that the wax or its precursors arrived at the surface by extrusion through the pores. On the other hand, Russian workers Kravkina & Miroslavov (1969) observed similar pores in relatively wax-free areas on leaf cuticles of *Triticum durum* and *Pseudosasa japonica*. More recent attempts to match surface pores with microchannels across the cuticle have been made by Hall (1967b) and by Fisher & Bayer (1972).

Using conventional thin section techniques for electron microscopy, Scott *et al.* (1958) provided evidence for pits in the outer epidermal cell wall. Subsequently, the work of Franke (1961, 1964, 1969) on 'ectodesmata' gave further support for the existence of a pathway from the protoplast to the cuticular surface. It does seem, however, that the structures Franke called 'ectodesmata' are confined to the outer epidermal wall and rarely if ever traverse the cuticle itself (Erwin &

Sikkema, 1971). There have been other studies in which penetration of cuticles with fine fibrils has been described (Schnepf, 1963; Maier, 1968; Seivers, 1968).

In extending the scope of the earlier investigations reported in this study (Chapter 1) to examine continuity of microchannels across the whole epidermal wall and cuticle, several methods were tried. Included in these were (i) platinum-carbon shadowing of thin sections, and (ii) incorporation of lanthanum during fixation (Revel & Karnovsky, 1967; Rowley & Flynn, 1971). Neither of these resulted in demonstrating microchannels, so that a freeze-fracture study was then initiated in the hope that this would avoid some of the artifacts associated with the conventional fixation-dehydration-embedding preparations. Though the freeze-fracture/freeze-etching technique pioneered by Steere (1957), Moor *et al.* (1961), Moor & Mühlethaler (1963), and Koehler (1968) has found increasing use in biological research, little of the work has been directed towards solving the problem of microchannels in the outer epidermal wall and cuticle other than the short report of Hall (1967b). Certainly a comparison between the freeze-etched image of fresh and fixed tissue was considered necessary.

METHODS

(a) Surface Replicas

A method similar to that employed by Hall & Donaldson (1962) was used. Leaves of *Brassica napus* grown under a series of temperature regimes were used for a comparative study together with those of the non-waxy mutant.

Whole leaves were submerged in chloroform for 30 minutes and given a final wash with fresh solvent after this time.

Sections of leaf were rapidly secured on a glass slide with IS 12 adhesive, LocTite (Ireland) Limited. The dewaxed leaf surface was subsequently shadowed with platinum-carbon and immediately flooded with 0.2% collodion in amyl acetate.

Increasing concentrations of collodion were flooded over the surface, allowing each previous layer to dry, until a strippable plastic film was formed.

The film was then stripped from the leaf surface and replicated with carbon as detailed in Chapter 1 for the preparation of carbon replicas. The collodion was removed with amyl acetate and the replica film given an overnight wash in acetone to remove all traces of collodion. The replicas were then examined directly in the electron microscope.

(b) Freeze-fractured Replicas

These were obtained by a method similar to that of Hall (1967b) being basically the freeze-etching technique of Moor & Mühlethaler (1963).

A 24/19 °C leaf was cut into 1.5 mm^2 pieces. These were floated, adaxial surface uppermost on (i) 40% glycerol (Moor, 1964, 1966) in 0.025 M sodium phosphate buffer for sixteen hours, or (ii) 3% glutaraldehyde in glycerol-buffer solution followed by a further eight hours in glycerol solution.

Tissue representing each treatment was placed on the edge of a copper disc such that the knife would fracture wax, cuticle, cell wall and protoplast. The tissue was immediately frozen in liquid Freon-22 (-150 °C) for 6 - 8 seconds after which it was transferred to liquid nitrogen for storage, prior to transfer to the freeze-etch unit.

In the Balzer freeze-etch unit (Balzer Model BA360M), the specimens were fractured, shadowed with Pt-carbon and replicated with a carbon film. The biological material was removed by the following procedure:

1. 45% sulphuric acid for six hours;
2. 70% sulphuric acid for six hours;
3. Concentrated calcium hypochlorite for six hours;
4. Wash in distilled water for one hour;
5. Acetone/water series to 100% acetone within 5 minutes;
6. Tetrachloroethane-phenol mixture (50:50) for 30 minutes.

(This step, though leading at times to loss of replicas through fragmentation, was necessary for removal of the cuticle which generally survived the acid/hypochlorite treatment.)

7. Transfer to acetone, followed by rehydration in acetone/water series to pure water.
8. Replicas picked up from water on coated 400-mesh grids.

RESULTS

Evidence of cuticular pores on the leaf surface of the non-waxy (gl_3) mutant, and on the dewaxed surfaces of waxy *Brassica napus* leaves, from different temperatures, is presented in Plates 2.1 and 2.2.

A replica taken from the surface of a smooth collodion film is shown as a control in Plate 2.3. This indicates a total absence of any similar structure which could be misinterpreted as being surface pores.

The non-waxy (gl_3) leaves were found to have a central pore diameter of 7 nm and an outer diameter of 54 nm. The cuticular pores on the waxy leaves, irrespective of growing temperature, possess a central pore diameter of 7 nm also and an outer diameter of 38 nm. These dimensions have been calculated from high magnification (× 131,000) plates.

Justification for the interpretation of the presented 'pores' being non-artifactual has already been given by Hall (1967a).

The freeze-fractured studies on the outer epidermal and non-epidermal cell wall region of *Brassica* are presented in Plates 2.4 a,b & c and 2.5a,b. Preservation of the chemically fixed tissue was only marginally superior to that of rapidly frozen fresh tissue.

Plates 2.4a and 2.4b represent fractures of the outer epidermal cell wall after overnight glycerolation and fixation with 3% glutaraldehyde. Plate 2.4a represents a cell wall in the centre of an epidermal cell and Plate 2.4b a cell wall junction between two such epidermal cells. Plate 2.4c illustrates higher magnification detail of the cell wall.

Plates 2.5a and 2.5b represent outer epidermal and non-epidermal cell wall fractures respectively following glycerolation. The relatively fine granular lamellae of non-epidermal cell walls were in consistent contrast with the rather coarse granular appearance of the outer epidermal cell wall. However, no system of microchannels could be demonstrated unequivocally in either type of cell wall.

DISCUSSION

In this study on *Brassica napus* the central perforation and the outer pore diameter of cuticular pores agree closely with the dimensions reported by Hall (1967a) for *Brassica oleracea*, and similar pores were also revealed on all other plants examined by him, including peas, clover, wheat, apple fruit and *Eucalyptus* spp.

The presence of cuticular pores can be established only by the dewaxing of leaf surfaces in solvent for some critical time, the period differing with species (Hall, 1967a). If all the surface wax is dissolved, the details of pore structure are difficult to demonstrate on the replicas. On the other hand, if dewaxing is not adequate, the wax remaining covers the cuticular surface and masks the presence of pores.

Examinations of numerous electron micrographs has so far failed to indicate any consistent difference in the spatial arrangement or the frequency of pores from different temperature treatments. Occasionally, distant rows of pores were observed, but it would seem that this feature largely reflected preferential removal of wax from that area of the cuticle rather than any significant change in distribution.

It has been suggested in the literature (Hall & Donaldson, 1962; Hall, 1967a) that one pore may give rise to one wax rod, with several hundred pores contributing to a single large wax plate. From the present study there would appear to be too great a discrepancy between the number of wax rods and the number of cuticular pores per unit area to support a one pore-one wax rod hypothesis, even taking into account that some pores may be non-functional.

The non-waxy (gl_3) plant exhibited a prominent array of cuticular pores, and yet no structural wax at all is present on the leaf surface of this plant. Apart from a marginally larger outer diameter, the frequency and spatial distribution of these pores resembled that of the waxy leaves in all other respects. Thin sections indicated that the upright structural wax (W_S, Plate 2.6) was rarely in direct contact with the cuticle. Rather, a sheet of non-structural wax (W_{NS}) was frequently seen to lie between the cuticle and the structural wax zone. It is possible therefore, that the frequency and/or the distribution of cuticular pores may have little to do with the ultimate structure of the wax.

The qualitative and quantitative differences in leaf waxes from plants grown at different temperatures (Whitecross & Armstrong, 1972) has not been shown in this study to be correlated with the frequency, the size or the spatial distribution of cuticular pores. This would completely nullify Hall's suggestion that "when cuticular stresses are produced the pore distribution is radically altered and fresh wax exuded on the surface by this different distribution causes a different form of wax deposit." (Hall, 1967a)

From the evidence presented in this study it seems more likely that the cuticular pores merely provide an exit for wax, presumably in solution or some liquid form to reach the leaf surface. Once there, the wax will have its final structure determined by other factors such as environmental conditions or chemical composition. These latter factors are examined in Chapters 3 and 4 of this study.

This study, though confirming the presence of cuticular pores on the leaf surface, together with the previous demonstration of microchannels across the cuticle of *Brassica napus* (Chapter 1), has still not been able to demonstrate wax microchannels within the cell wall, even in high magnification studies.

Hall (1967b) suggested the presence of 'microchannels' in the freeze-etched epidermal cell wall regions of clover, pointing out their similarity in dimension with the size of cuticular pores observed on the surfaces of dewaxed cuticles. These 'microchannels' structurally resembled the cuticular pores as seen on the cuticular surface, in spite of the fact that the freeze-fracture study presented a view of the cell wall at approximately 90° to the leaf surface.

The only evidence in the present study which might be interpreted as a microchannel system within the cell wall and having a radial orientation is illustrated in Plate 2.4b, designated A. If a microchannel through the cell wall were associated with each cuticular pore one would have expected numerous 'channels' of this type to be observed. This should be so even though changes in fracture planes and deviations of microchannels would naturally prevent one from observing the full length of a microchannel from cuticle to plasmolemma.

The consistently coarse granular lamellae of the epidermal cell walls (Plate 2.5a) as compared with a non-epidermal cell wall (Plate 2.5b) may indicate a looser bonding of the cellulose fibrils for the former. There may thus be an effective, though less discrete, diffusion pathway for the movement of wax from the plasmolemma to the cuticle.

In view of the poor evidence to date, from this and previous studies, for the existence of microchannels in epidermal cell walls, it is postulated that wax in some liquid form diffuses more or less in bulk from the plasmolemma to the cuticle, where it subsequently reaches the cuticle surface via a series of pores whose distribution is seen to be largely random.

Plates 2.1 - 2.6

The following plates illustrate the results for waxy *Brassica napus* plants unless specifically stated otherwise.

The preparation of replicas of dewaxed leaf surfaces involved Pt/C shadowing and two-stage replication using collodion and carbon film.

Freeze-fractured replicas were prepared by Pt/C shadowing and carbon replication.

The thin section material (Plate 2.6) was prepared by glutaraldehyde/osmium tetroxide fixation and uranyl acetate and lead citrate staining.

Dimension lines on micrographs represent 1 μm except where indicated otherwise.

Plate 2.1: Electron micrographs illustrating 'pores' on the chloroform dewaxed leaf surface of

(a) a non-waxy (gl_3) mutant plant.

(b) a 15/10 °C grown plant.

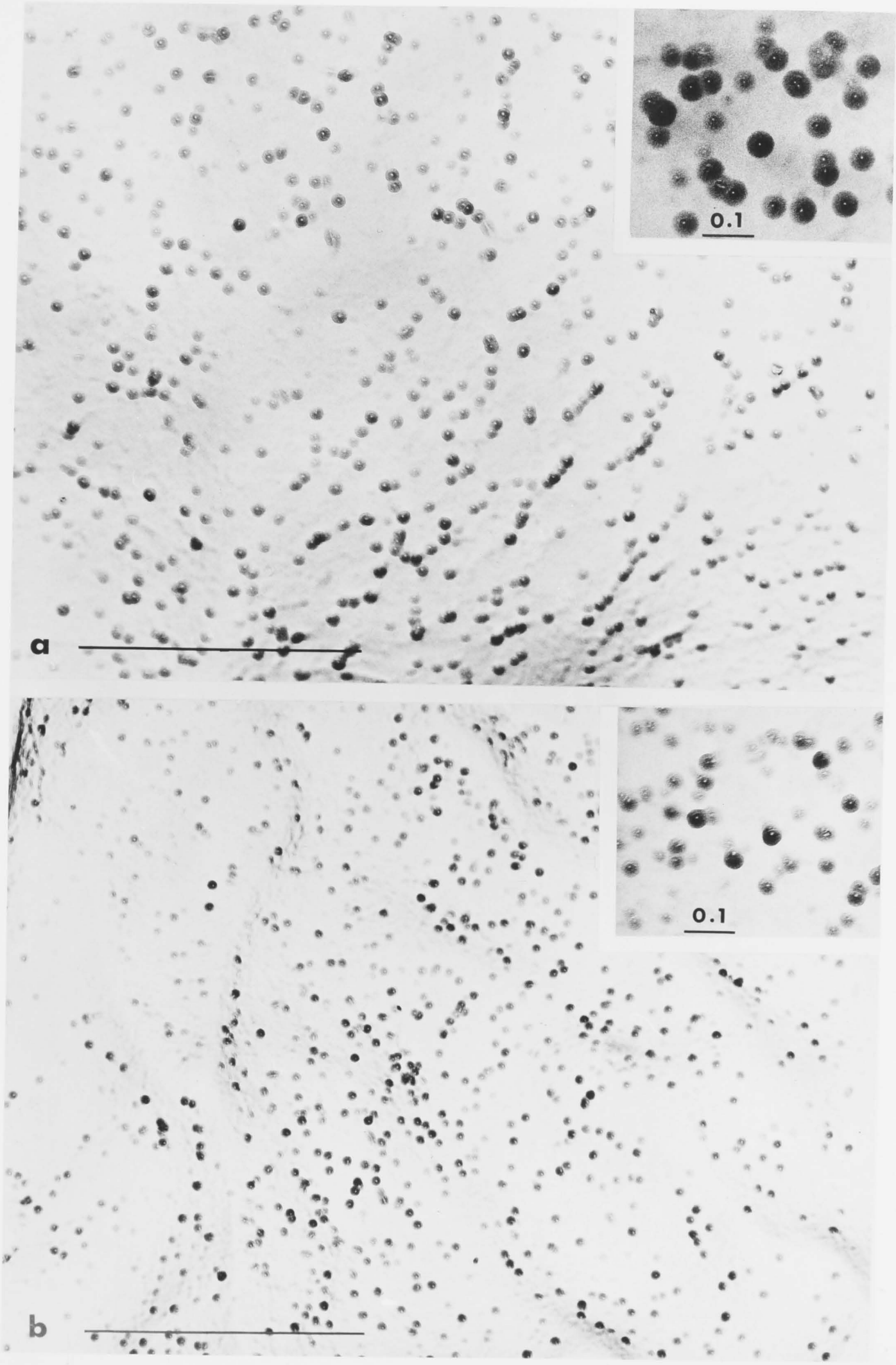
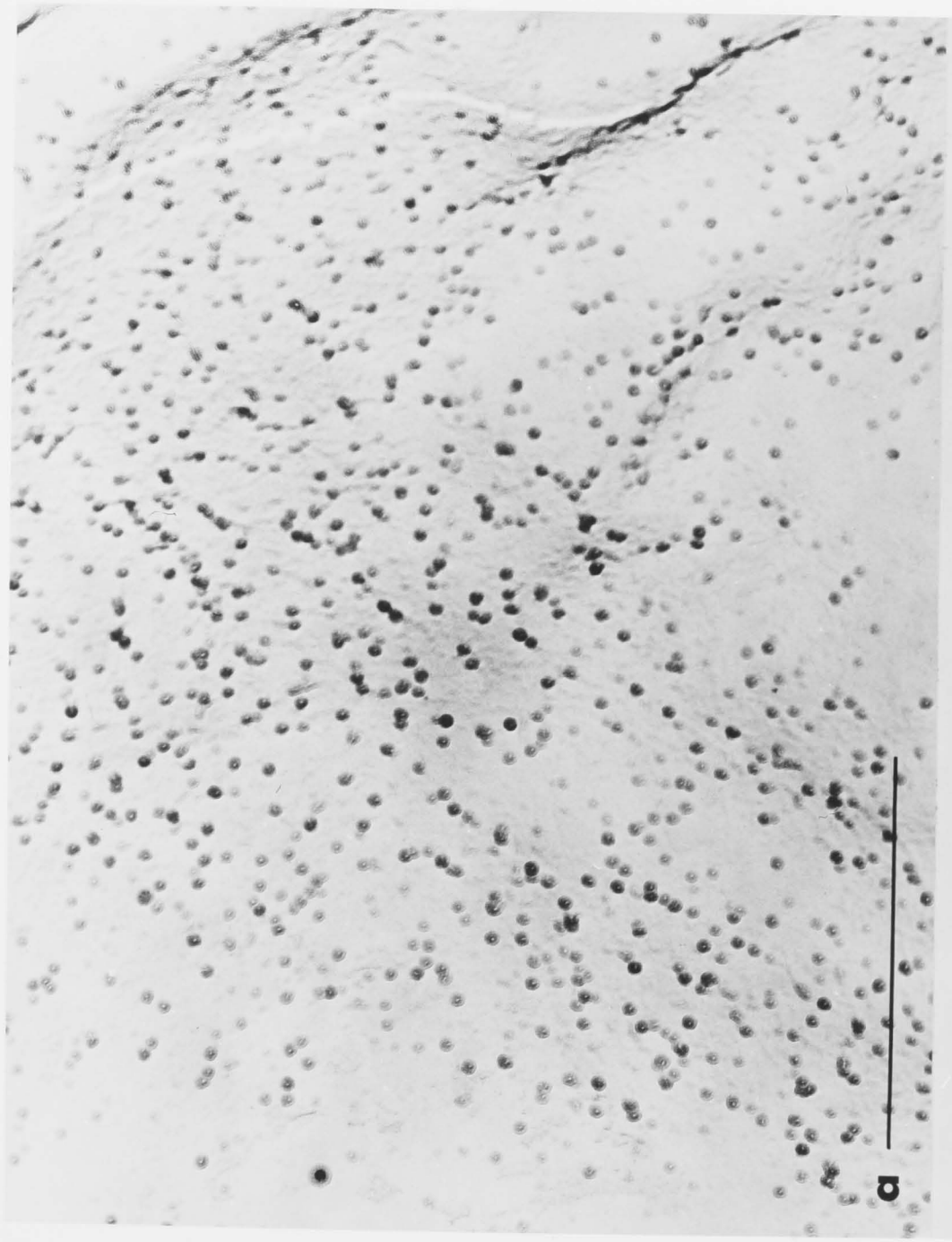

0.1
a
0.1
b

Plate 2.2: Electron micrographs illustrating 'pores' on the chloroform dewaxed leaf surface of

(a) a 24/19 °C grown plant.

(b) a 36/31 °C grown plant.


a

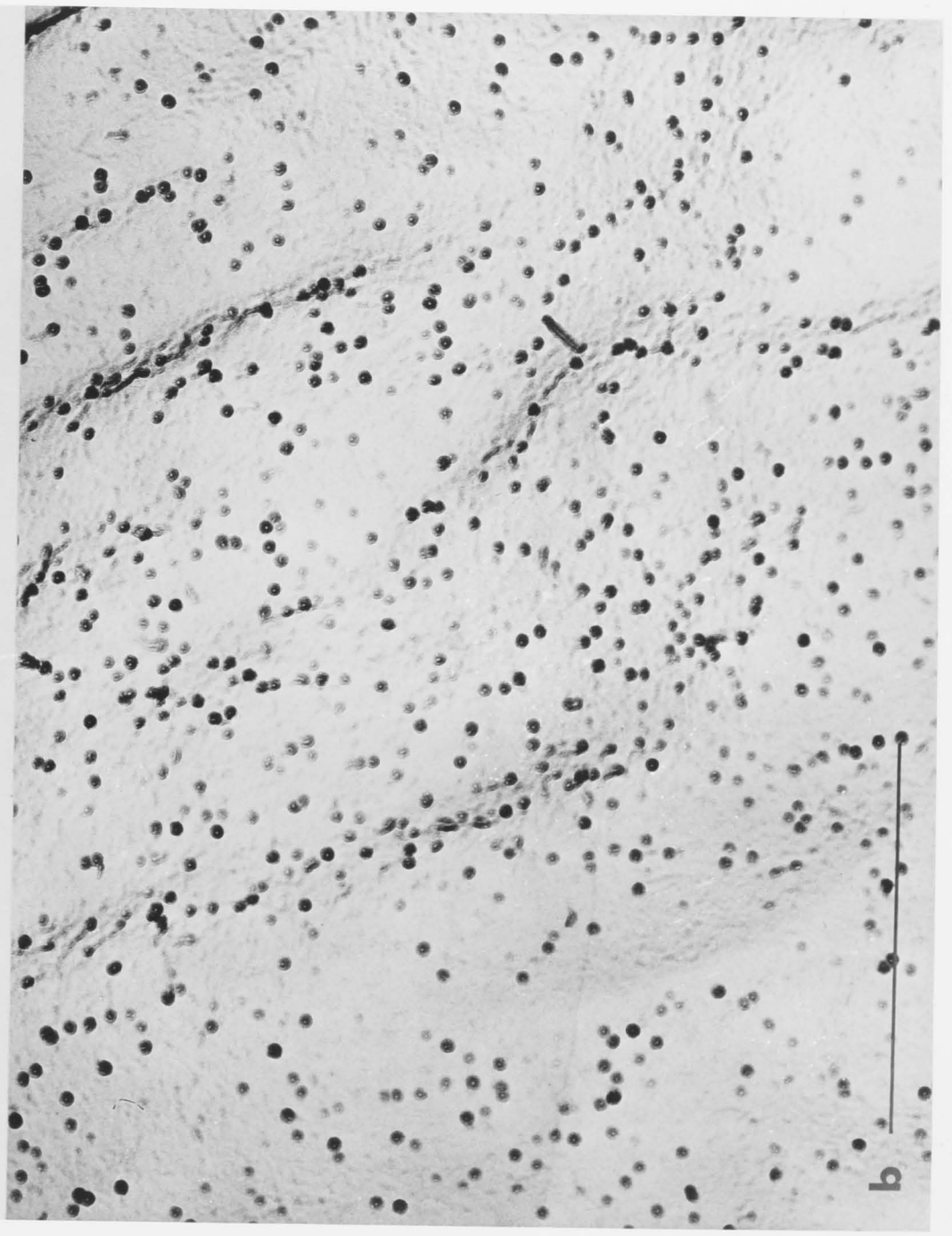

b

Plate 2.3: Electron micrograph illustrating the surface of a smooth parlodion film.

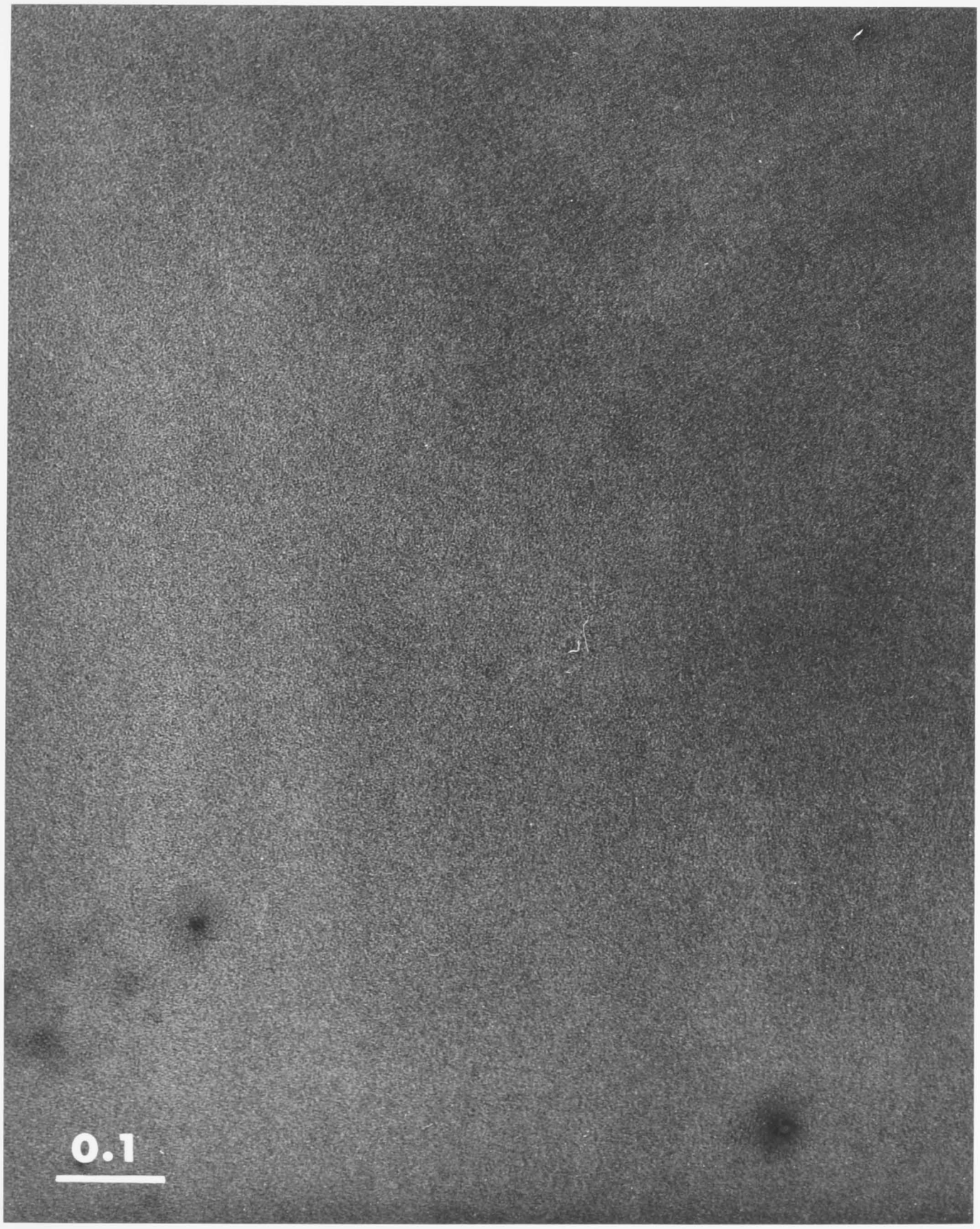
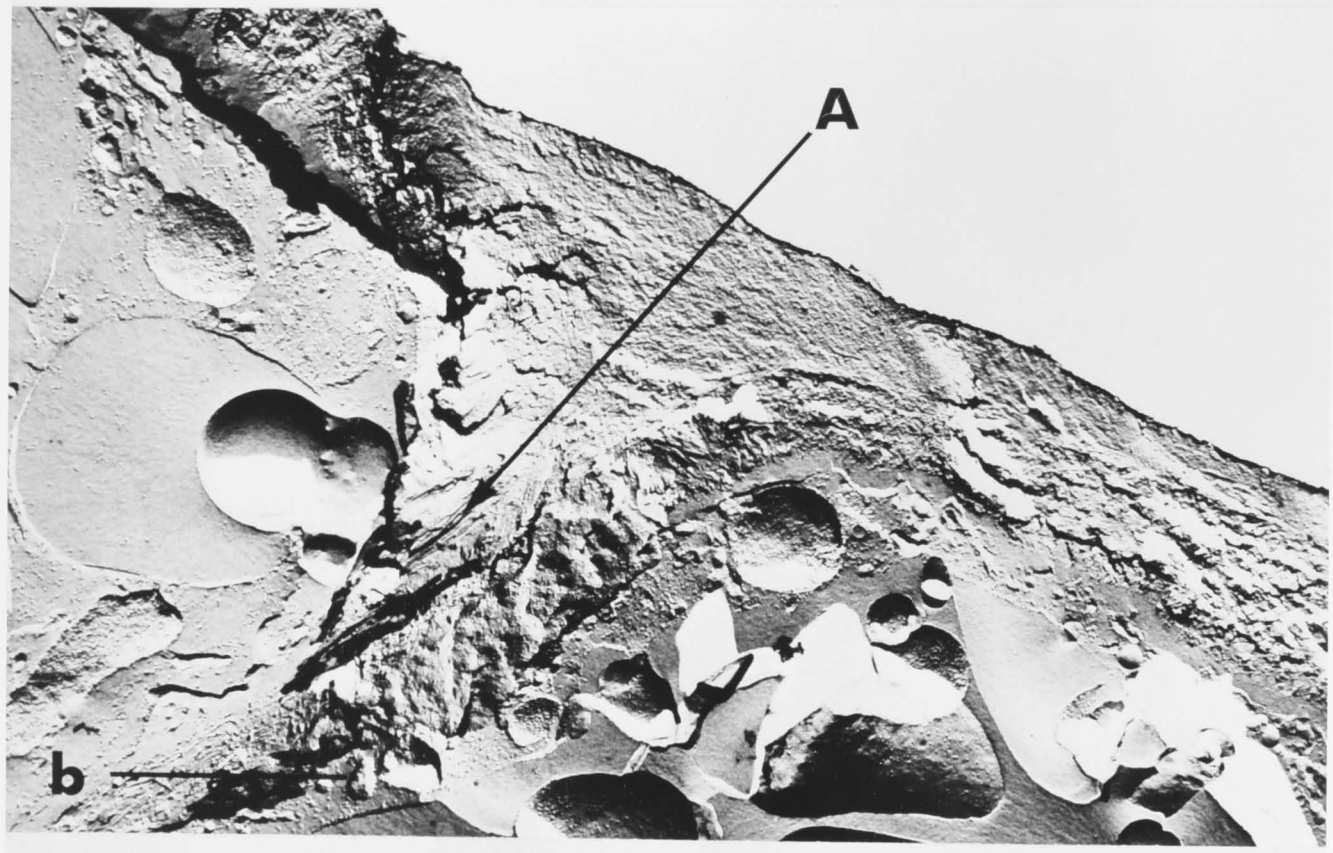

0.1

Plate 2.4: Electron micrographs illustrating freeze-fractured replicas of

(a) an outer tangential epidermal cell wall.

(b) a cell wall margin.

(c) a tangential cell wall at high magnification.


A
b


c

Plate 2.5: Electron micrograph illustrating freeze-fractured replicas of

(a) an outer tangential epidermal cell wall.

(b) a non-epidermal cell wall.

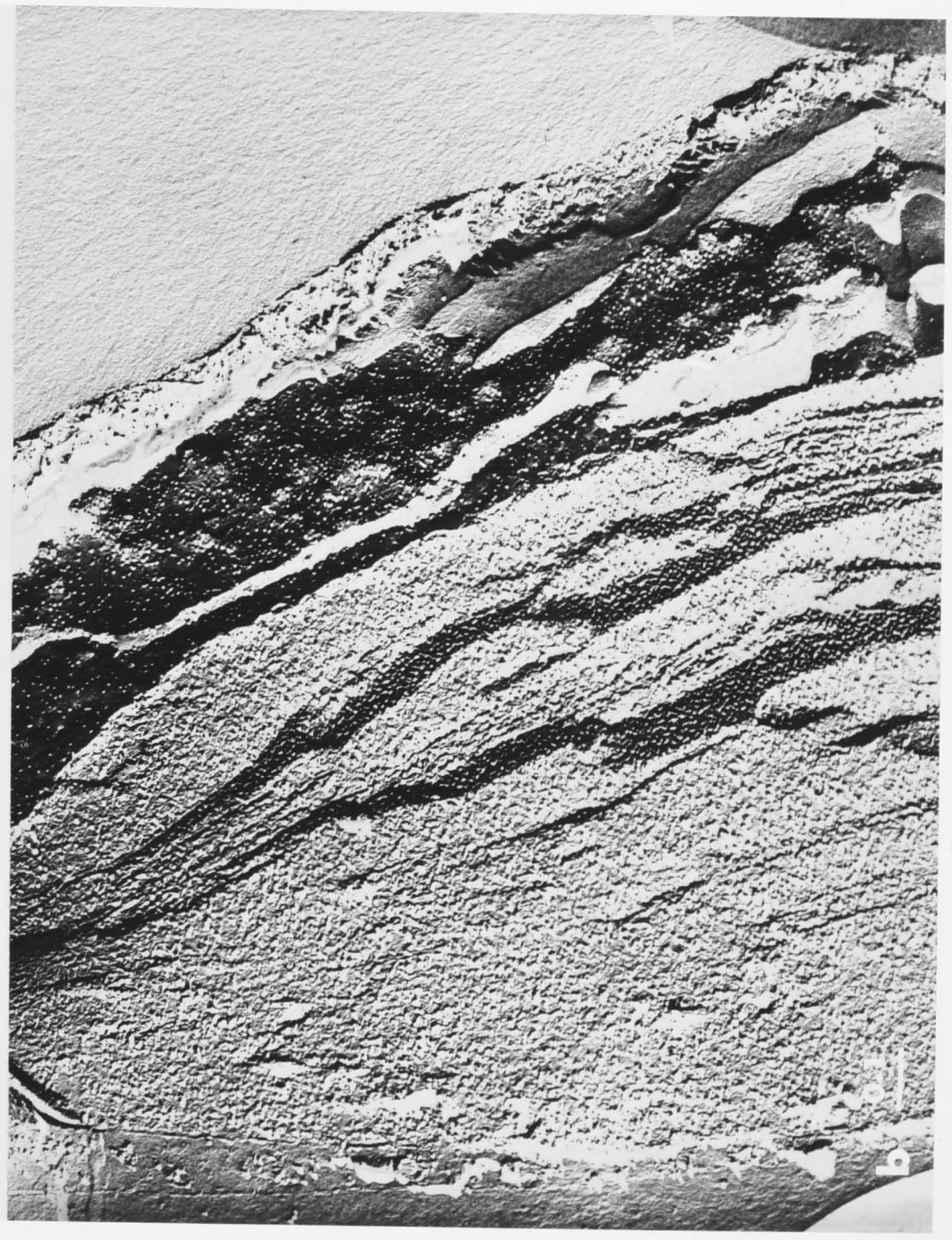


Plate 2.6: Electron micrograph illustrating a non-structural wax layer (W_{NS}) as an apparent substrate for the structural wax layer (W_S).


W_S
W_{NS}

CHAPTER 3

CHAPTER 3

EFFECT OF TEMPERATURE ON THE CHEMISTRY OF LEAF SURFACE WAXES

INTRODUCTION

The occurrence of wax in plants, though primarily on the surfaces of leaves, fruits and stems, is not confined to these regions. Smaller quantities have been reported in bark (Swan, 1963; Kurth, 1967), in flowers (Hemming *et al.*, 1963; Mecklenburg, 1966; Wollrab *et al.*, 1967), in roots (Sharma & Gupta, 1966; Akperbekova, 1967), in wood (Pearl & Happocks, 1961; Bell & Harvey, 1963; Grice *et al.*, 1968), in seed coats (Carncluff *et al.*, 1966), and in cell organelles (Gülz, 1968). Nevertheless, most attention has been directed towards the waxes occurring on the superficial layers, and a high proportion of these is made up of long chain n-primary alcohols, n-ketones and n-paraffins. Reference is made however to Martin & Juniper (1970, table 3.1, pp.64,65) summarising the major wax constituents of many plant species. A great diversity of minor constituents also occurs, including alkenes (Šorm *et al.*, 1964), branched hydrocarbons (Downing *et al.*, 1960; Mold *et al.*, 1963; Wollrab *et al.*, 1965; Eglinton *et al.*, 1966; Brieskorn & Feilner, 1968; Kaneda, 1968), aromatics (Horn & Lamberton, 1963; Nagy *et al.*, 1965), aldehydes (Lamberton & Redcliffe, 1960; Lamberton, 1965), flavones (Lamberton, 1964; Horn *et al.*, 1964), diols (Mazliak, 1962), β-diketones (Horn & Lamberton, 1962; Horn *et al.*, 1964; Tulloch & Weenink, 1966; Hallam, 1967; Hallam & Chambers, 1970; Barber & Netting, 1968), and even a seleniferous wax (McCulloch *et al.*, 1963).

The chemistry of leaf waxes of *Brassica* spp. has been intensively studied, beginning with the classical work of Channon & Chibnall (1929), Sahai & Chibnall (1932), and Chibnall & Piper (1934). More recent studies using modern chromatographic techniques include those of Purdy & Truter (1963b,c), Laseter *et al.* (1968), Macey (1970, and Macy & Barber (1970a,b). Differences in chemical composition have been shown to accompany physical differences in degree of leaf glaucousness in *Brassica* cultivars (Hall *et al.*, 1965; Macey & Barber, 1970b). On the other hand,

comparatively little work has been done on variations in wax composition resulting from varying growth conditions of a single species of cultivar. Macey (1970) studied the effect of light on wax synthesis in *Brassica oleracea* but no work has been done on the result of growth under different temperature conditions. It has already been shown (Whitecross & Armstrong, 1972) that the temperature effects on wax fine structure were far more dramatic than the light effects.

In order to gain evidence which might explain the observed temperature modifications in fine structure, it was decided to undertake analytical characterisations of waxes isolated from leaves grown under some of the standard temperature conditions used in the previous experiments (Chapter 1). In these analyses, changes in the occurrence of wax constituents was to be looked for, though these need not necessarily have been on a presence or absence basis. Even rather small differences in the proportions of various wax components could account for variations in the pattern of crystallisation of such mixtures (M.J.K. Macey, personal communication). Since it was possible then that rather small chemical differences might be encountered, it was therefore necessary to ensure that samples to be analysed should be isolated from leaf surfaces without contamination by internal lipids.

In previous wax removal procedures, Chambers & Possingham (1963) showed that comparatively drastic treatments were required for the complete removal of most waxy layers on grapeskins, while Hallam (1967) attempted a quantitative removal of *Eucalyptus* wax by melting it off the leaves with hot water (wax melting point below 70 °C, Barber, 1955). Solvent removal has given varied results. Martin & Batt (1958) and Purdy & Truter (1961) removed wax by immersing leaves for short periods in successive portions of ether at room temperature. Roberts *et al.* (1961) concluded that the amount of waxy material obtained depended largely on the solvent used. Martin (1960) and Roberts *et al.* (1961) concluded that most of the wax could be removed from leaf surfaces in the first of four ten-second washes in chloroform. Many others have followed this lead in using chloroform for wax removal (Dewey *et al.*, 1961; Dudman & Grncarevic, 1962; Eglinton *et al.*, 1962; Whitecross, 1963). Silva Fernandes (1964) and Hallam (1967, 1970c) have refluxed leaf samples over petroleum ether to isolate waxes.

In this investigation, an attempt was made to select a suitable standard procedure for wax removal. Attention was given to the solvent used and the duration of solvent treatment. The aim was to select a method which would reliably remove all the structural surface wax while at the same time cause absolute minimal internal disruption at the cell level. It was hoped that the contamination of samples with internal lipids would be considerably reduced if such a procedure were found.

METHOD

Isolation of Leaf Waxes

Leaf surface wax was isolated by individual dipping of undamaged leaves in two consecutive baths of redistilled n-hexane for a total of 8 - 10 seconds. Electron microscope studies showed all structural wax to be removed by this treatment (Plate 3.1a), internal cell disruption being minimal (Plate 3.1b) when compared with that caused by other solvent and/or washing procedures (Plates 3.2 a & b). Very young and fully expanded leaves were not sampled, thus avoiding any variation in composition due to age which might have masked the effects of a specific treatment.

The hexane extract solutions were bulked for each sample, filtered and most of the solvent removed in a rotary evaporator under vacuum at 25 °C. The concentrated wax solutions were evaporated to dryness and stored at 4 °C under oxygen-free nitrogen gas prior to analysis.

Thin Layer Chromatography

Kieselgel G was made into a slurry with distilled water (30 gm/60 ml) and spread onto 20 × 20 cm glass plates by means of a Shandon plate spreader (~ 250 µm thick). Plates were left to dry overnight, reactivated at 110 °C for two hours and cooled half an hour prior to use.

Five µl of a 2 - 3% wax in chloroform solution was spotted onto the plate. Development of the chromatogram was by the ascending-solvent technique using benzene as the solvent. Separated wax components were located by spraying the plate with concentrated sulphuric acid as a chromogenic reagent and heating to 160 °C for ten minutes.

Infra-red Spectroscopy

Infra-red spectra of total wax were obtained by scanning an

evaporated film of wax on sodium chloride discs in the 625-4000 cm^{-1} range.

Gas-liquid Chromatography

Waxes grown at three different temperatures (15/10 °C, 21/16 °C and 36/31 °C) were analysed with a Varian Aerograph 2100 gas chromatograph using a thermal conductivity detector. Temperature programming was utilised in the 150 °C - 270 °C range at 2° per minute. A 3% OV-101 column was used with nitrogen as the carrier gas at the rate of 40 ml/min. Individual fractions were quantified by means of a continually monitoring integrator.

Mass Spectrometry

Mass spectra were run on a GEC - AEI MS902 instrument using an electron energy of 70 V. Samples were introduced on a direct insertion probe and run at source temperatures of 80 °C - 350 °C. Mass spectral data were fed into a direct on-line computer link. Empirical formulae were obtained on a direct print-out, masses being calculated from atomic weights given by Mattauch & Thiele, 1964 atomic mass table, *Nuclear Physics* 67, 1-31 (1965).

RESULTS

Thin Layer Chromatography and Infra-red Spectroscopy

Plate 3.3 illustrates a comparison between waxes from three different temperature treatments (15/10 °C, 21/16 °C and 36/31 °C) as analysed by thin layer chromatography. The separated components were chemically classified on the basis of infra-red spectroscopy and Rf values. Waxes from all three temperatures were found to include hydrocarbons, ketones, aldehydes, esters, primary and secondary alcohols, and ketols. These results were virtually identical to those reported by Purdy & Truter (1963b,c) for *Brassica oleracea* leaf waxes.

An infra-red spectrum for total wax from the 15/10 °C condition is shown in Plate 3.4 as an example of the type of data obtained from all three parent waxes.

G.L.C. and Mass Spectrometry

Plate 3.5 illustrates the most significant proportionality differences in components of 15/10 °C, 21/16 °C and 36/31 °C waxes as

resolved by G.L.C. A consistent trend was observed throughout the series, in that increased growing temperature resulted in a significant reduction of the C_{29} and C_{31} hydrocarbons and a marginal increase in the C_{29} ketone. The C_{29} and C_{31} hydrocarbons constituted more than 95% of the total hydrocarbon content of the wax. The ratio of C_{29} to C_{31} hydrocarbon was observed to be 3.7:1 for 15/10 °C and 21/16 °C waxes decreasing however to 2.8:1 for the 36/31 °C wax.

Plate 3.6 illustrates part of a mass spectral trace for a total wax sample. Mass spectra confirmed the G.L.C. result that all three waxes had the same structural components. Unlike the G.L.C. data, mass spectrometry produced qualitative composition data only. The two major hydrocarbons were found to be normal straight chain ones having the following respective parent ion peaks C_{29} m/e 408 and C_{31} m/e 436.

It is known that ketones frequently undergo rearrangements followed by fragmentation with retention of the + charge on the oxygen containing fragment (Laseter *et al.*, 1968). In such a case, one would expect a peak at m/e 240 and then by a double rearrangement a peak also at m/e 182 as observed. Such spectral characteristics could only result from a symmetric ketone having 29 carbon atoms, specifically 15-nonacosanone.

The mass spectra also clearly and reproducibly indicated the presence of a series of long chain ester compounds having the following respective parent ion peaks C_{42} m/e 620, C_{43} m/e 634, C_{44} m/e 648, and C_{45} m/e 662. These have never been reported from any previous studies on waxes of this type.

DISCUSSION

Previous workers concerned with the isolation of *Brassica* spp. leaf surface waxes, while using standard solvent treatments giving reproducible results, have still not convincingly demonstrated that their wax residues did not contain internal contaminants (Purdy & Truter, 1963a; Silva Fernandes *et al.*, 1964).

The procedures adopted in this study, while appearing perhaps somewhat over-cautious, have been shown to remove all structural leaf waxes with negligible internal cell disruption. While electron microscopic studies showed chloroform to be a more effective solvent in removing the sub-structural surface wax adjacent to the cuticle in *Brassica* spp., quantitative removal of surface wax for chemical analysis

was of no significance in this study. Selective solvent extraction of wax components was not ever encountered. This study was concerned with a possible chemical correlation with the fine structural phenomena reported in Chapter 1 and thus it was more important to remove just the structural wax.

Where very small differences in the proportion of components is sought it was also considered essential to use freshly isolated waxes stored in an inert atmosphere prior to analysis. Within the literature there are few reports on the conversion and inter-conversion of wax components, although Morozova *et al.* (1970) noted changes in the chemical composition of apple wax during storage under atmospheric conditions.

The use of thin layer chromatography and infra-red spectroscopy are simple and rapid means of determining the functional groups of leaf surface wax (Purdy & Truter, 1963b,c; Holloway & Challen, 1966; Eglinton & Hamilton, 1967; Hallam & Chambers, 1970). The chemical components of *Brassica napus* leaf wax have been shown to resemble closely those of other *Brassica* spp. both quantitatively and qualitatively (Purdy & Truter, 1963b,c; Barber & Macey, 1970b).

From this present study, it has been clearly established that increasing the growing temperature of *Brassica napus* had the consistent effect of reducing the amounts of both C_{29} and C_{31} hydrocarbons. Accompanying these hydrocarbon reductions, the proportion of the symmetric C_{29} ketone (15-nonacosanone) has been shown to increase slightly.

The use of a comprehensive range of growing temperatures, in previous sections of this work, has demonstrated that increased plant growing temperature causes a transition from a rod-type to plate-type wax. No controlled method of wax extrusion from the cuticle surface has been determined for the different temperature grown plants to account for the variation in wax pattern. In view of these findings, it is now suggested that plate wax formation may be induced by a reduction in the total hydrocarbons and an increase in the ketone proportion.

The ratio of C_{29} to C_{31} hydrocarbons has been shown in this study to be 3.7:1 for the 15/10 °C and 21/16 °C growing temperatures and 2.8:1 for 36/31 °C. Hill & Mattick (1966) obtained a ratio of 2.4:1 for these two hydrocarbons in *Brassica oleracea*. The hydrocarbon ratio may be of less

consequence than the ketone variation in the pattern of wax formation, since the same C_{29} to C_{31} hydrocarbon ratio was obtained in both rod-type (15/10 °C) and plate-type (21/16 °C) waxes.

It was not possible to demonstrate the presence of a particular wax component associated with a particular wax type as noted by Hallam & Chambers (1970). In that study an excellent correlation was found between rod-type wax and the presence of a β-diketone component in the wax of *Eucalyptus* spp. The fact that not even the slightest trace of a β-diketone component could be found in any *Brassica napus* wax suggests that no one wax component determines the final wax pattern for plants in general. The results of this study rather indicate that small but consistent differences in two or more major wax compounds are likely to be responsible for the differences in the ultimate wax pattern. Further, it seems apparent that synthesis of the hydrocarbon and ketone components at least is controlled in part by a temperature sensitive system.

In conclusion, it is pointed out that the mass spectrometer used in this study was capable of detecting compounds up to a formula of $C_{50}H_{100}O_3$ making it clearly capable of evaluating high molecular weight compounds of the type likely to be found in plant waxes. An example of this greater resolution has been the demonstrated presence of C_{42}, C_{43}, C_{44} and C_{45} esters in *Brassica* wax, here reported for the first time.

Plates 3.1 and 3.2

The following plates illustrate the results for waxy *Brassica napus* plants only.

The micrograph of a dewaxed leaf surface was prepared by Pt/C shadowing followed by carbon replication.

The thin section material was prepared by glutaraldehyde/osmium fixation, and uranyl acetate and lead citrate staining.

Dimension lines on micrographs represent 1 μm except where indicated otherwise.

Plate 3.1: Electron micrographs illustrating the effect of an 8 second wash in n-hexane on an intact 24/19 °C grown leaf.

(a) The leaf surface.

(b) The outer tangential epidermal cell wall-cytoplasm region.


a
b

Plate 3.2: Electron micrographs illustrating the effect on the outer tangential epidermal cell wall-cytoplasm region after washing intact 24/19 °C grown leaves in

(a) chloroform for 8 seconds.

(b) n-hexane for 5 minutes.

These micrographs to be compared with Plate 3.1b.

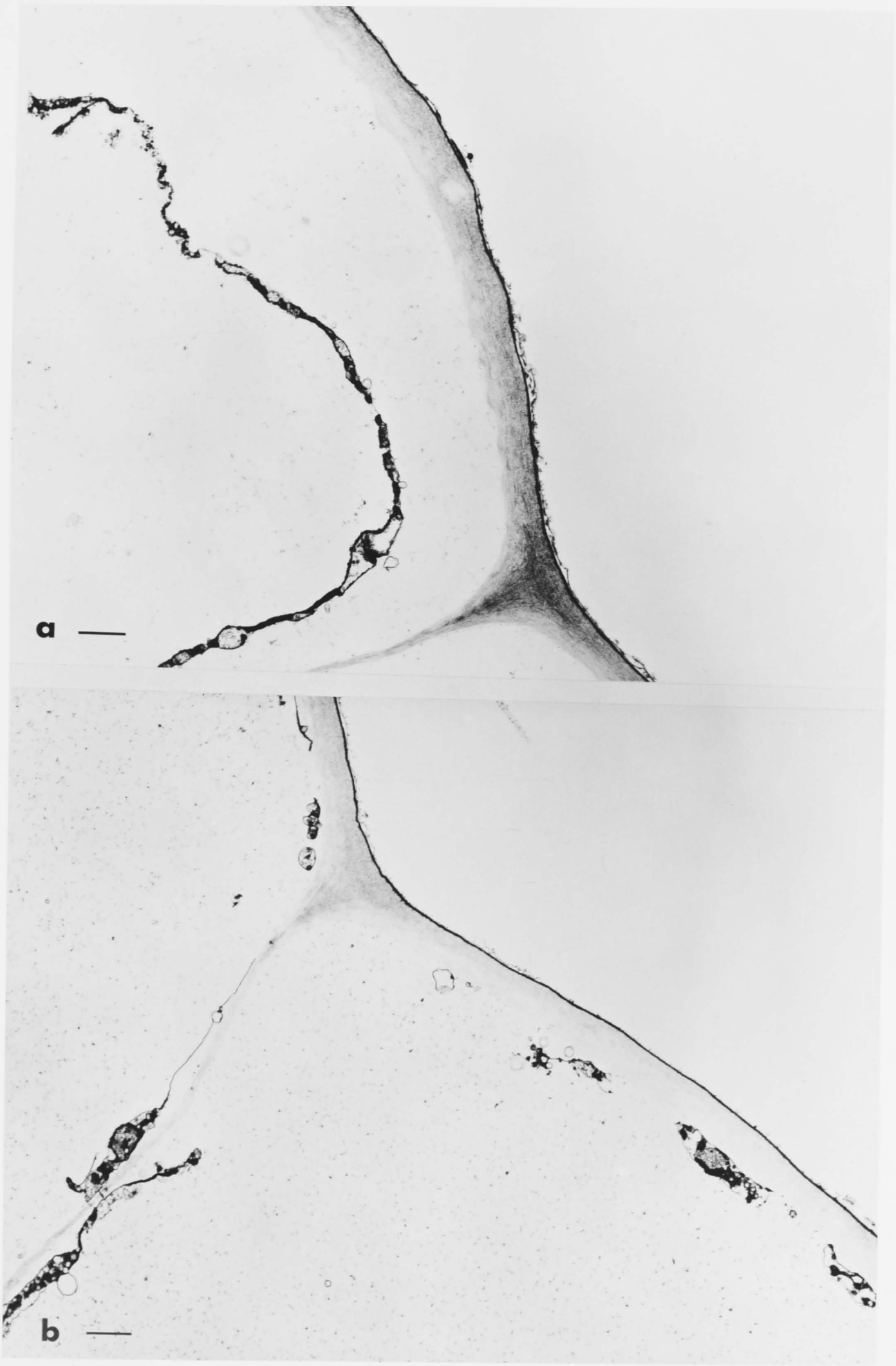

a
b

Plate 3.3: Thin layer chromatograph illustrating a comparison between waxes isolated from three different growing temperatures (15/10 °C, 21/16 °C, 36/31 °C). A - fatty acids; B - primary alcohol; C - ketol; D - secondary alcohol; E - aldehyde; F - ketone; G - ester; H - hydrocarbon.

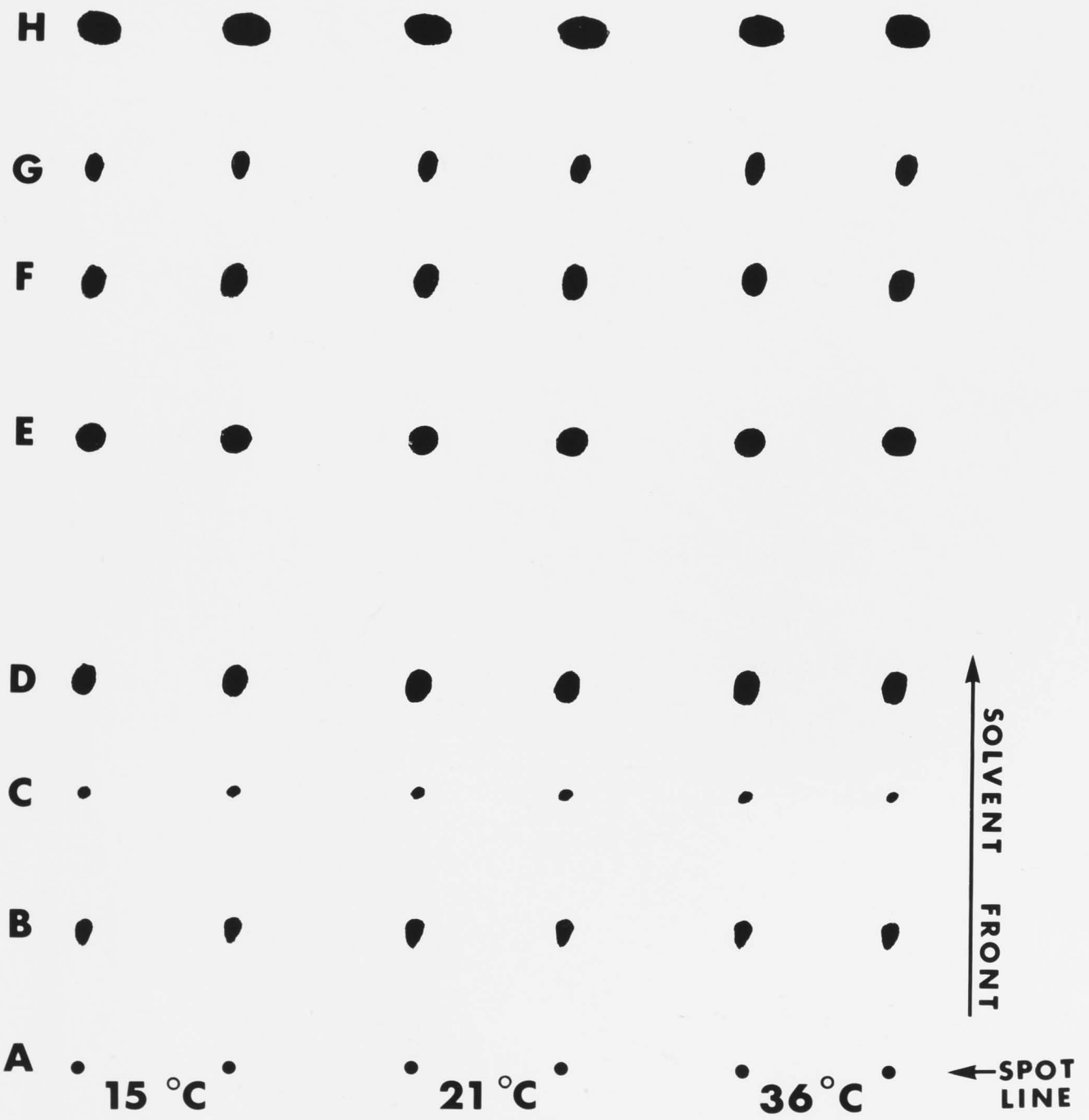

H
G
F
E
D
C
B
A
15 °C
21 °C
36 °C
SOLVENT FRONT
SPOT LINE

Plate 3.4: Infra-red spectrum for total wax for a 15/10 °C temperature grown plant.

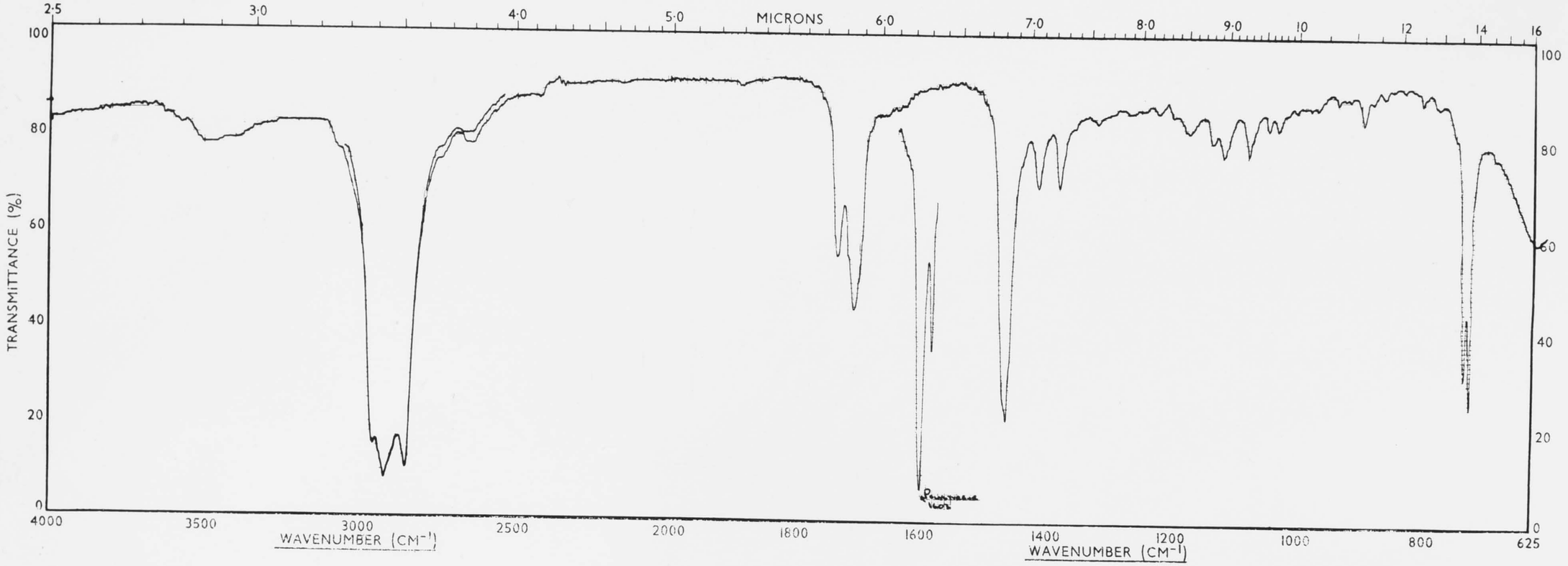

2·5
3·0
4·0
5·0
MICRONS
6·0
7·0
8·0
9·0
10
12
14
16
100
80
60
40
20
0
TRANSMITTANCE (%)
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
625
WAVENUMBER (CM⁻¹)
WAVENUMBER (CM⁻¹)

Plate 3.5: Histogram illustrating the most significant proportionality differences in components of 15/10 °C, 21/16 °C and 36/31 °C waxes as resolved by G.L.C. Each fraction was quantified by means of a continually monitoring integrator.

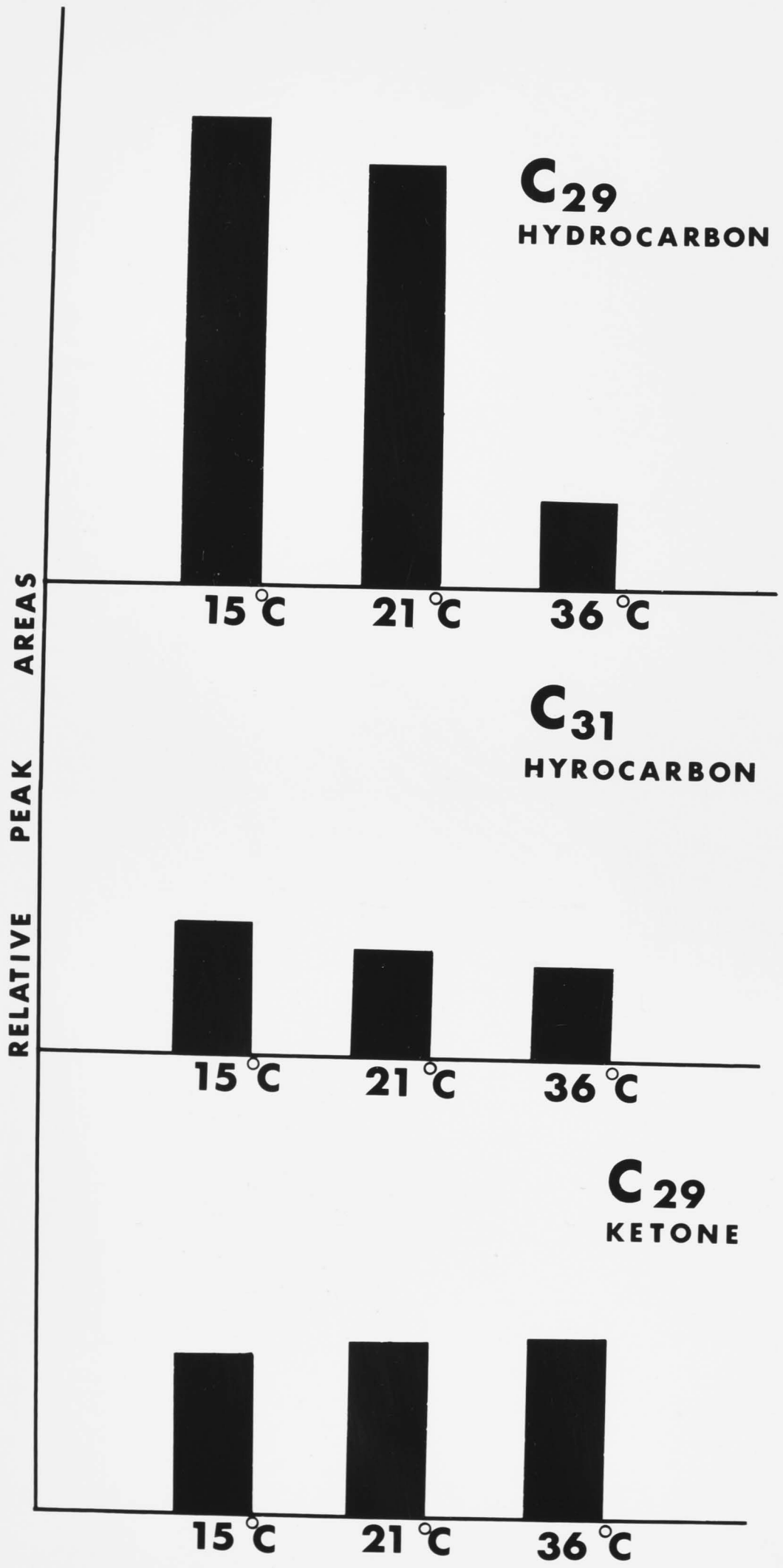
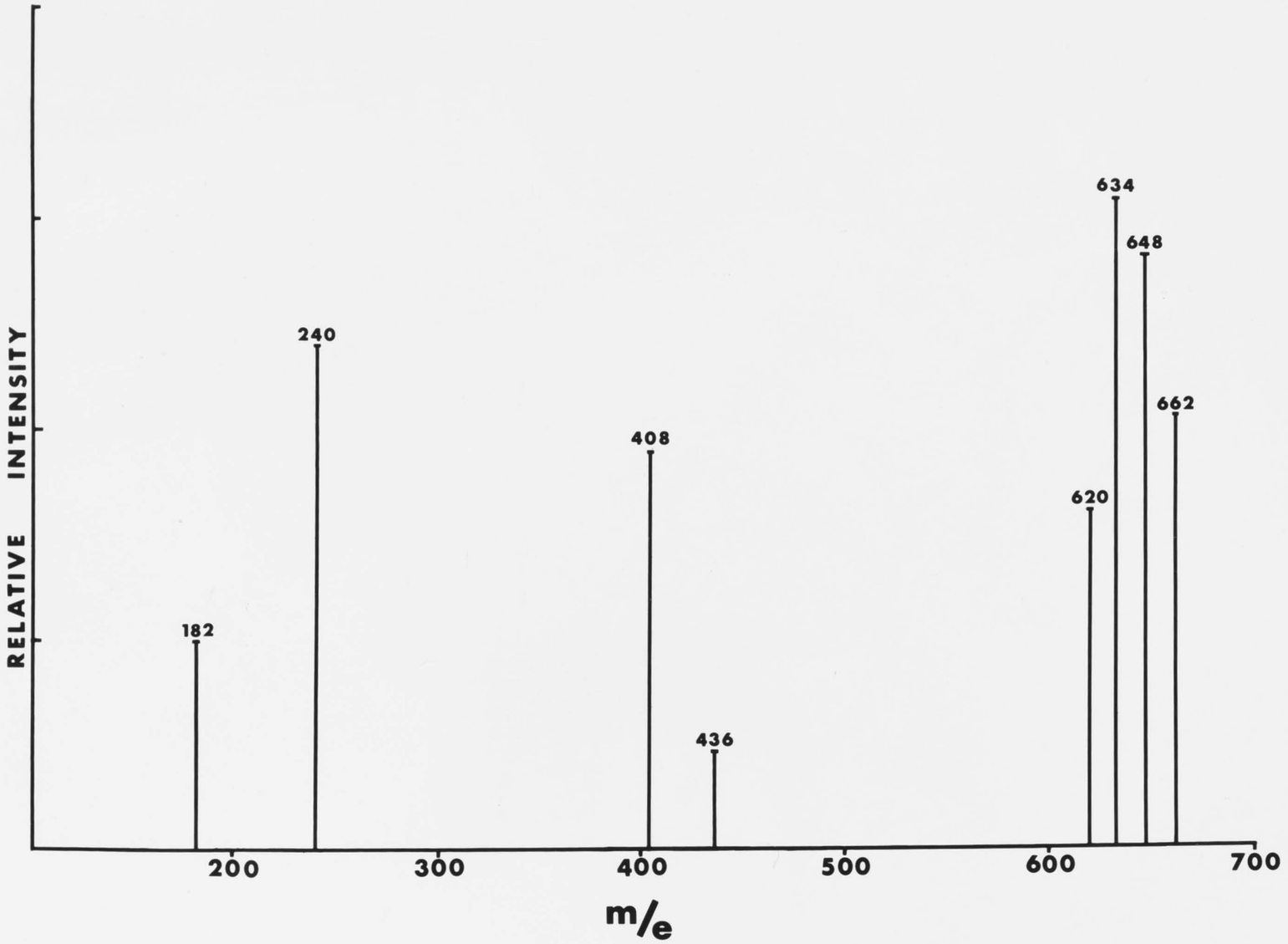

RELATIVE PEAK AREAS
C_{29}
HYDROCARBON
15 °C
21 °C
36 °C
C_{31}
HYROCARBON
15 °C
21 °C
36 °C
C_{29}
KETONE
15 °C
21 °C
36 °C

Plate 3.6: Mass spectra of C_{29} and C_{31} hydrocarbon, C_{29} ketone (15-nonacosanone) and C_{42-45} esters.


RELATIVE INTENSITY
182
240
408
436
620
634
648
662
200
300
400
500
600
700
m/e

CHAPTER 4

CHAPTER 4

RECRYSTALLISATION STUDIES ON HIGH AND LOW TEMPERATURE WAXES

INTRODUCTION

Three separate factors, individually or in combination, have been suggested as influencing the pattern of structural wax on leaf surfaces. These factors are:

(i) the chemical composition of the wax itself;

(ii) the means by which the wax is extruded on to the leaf surface; and

(iii) the environmental conditions under which the ultimate crystallisation of the extruded wax takes place.

Each of these factors has, to some extent, been dealt with in previous chapters. In Chapter 1, and in previous studies (Whitecross, 1963; Whitecross & Armstrong, 1972), the environmental conditions, and more particularly temperature conditions, were shown to have an appreciable influence on the fine structure of *Brassica* leaf waxes. In Chapter 2, evidence was presented suggesting that microchannels and cuticular pores, though the probable pathway for wax extrusion to the surface, were unlikely to determine the shapes of wax deposits as seen in the electron microscope. Thirdly, in Chapter 3, it was shown that the growth conditions which produced the variations in fine structure of the surface waxes also produced consistent, though small differences in the proportions of some chemical components of the waxes.

There have also been reported attempts to correlate the appearance of wax structures on the leaf surface with the crystallisation behaviour of wax isolated from leaves and allowed to come out of solution under experimental conditions. Whitecross (1963) recrystallised cauliflower wax, derived from high and low temperature plants, under a variety of conditions. He concluded that the waxes were chemically very similar and that the principal effect of environment was to vary the rate of crystal formation, giving rise to quite marked differences in fine structure.

Hallam (1967), working with various species of *Eucalyptus*, inclined to the view that leaf wax pattern was largely dependent upon the presence of certain chemical components in the wax.

Rod-type wax was shown to correlate well with the presence of β-diketones (e.g. in *E. globulus*), while plate-type waxes lacked these characteristic substances. In recrystallisation studies on isolated waxes from *Eucalyptus* spp., Hallam (1970c) showed that the rod structure of *E. globulus* wax and the plate structure of *E. ovata* wax persisted, confirming that their different fine structure probably resulted from their very different chemical composition.

Against this background, it was decided to investigate the recrystallisation behaviour of *Brassica napus* wax. Unlike the eucalypt waxes of Hallam (1970c), these waxes had only small quantitative differences in major constituents, yet the fine structure as modified by temperature conditions, manifested profound and consistent differences. It was decided to use, for this study, wax isolated from plants grown under the extreme temperature conditions imposed in the earlier experiments, viz. 15 °C and 36 °C. It was thought appropriate also to test the recrystallisation of both waxes largely at the two 'native' temperatures of 15 °C and 36 °C, but with variations within these treatments to allow differences in the rate of solvent dispersal.

Finally, in order to test whether the structural wax of *B. napus* does indeed result from a crystallisation process, subsequent to extrusion of amorphous wax, an examination of small apical leaves was undertaken. This was to enable a comparison between wax present, at this very early stage with that found on expanded leaves. If a progression were to be found from amorphous wax to structured wax, crystallisation could be presumed. If, on the other hand, structured wax were found in very young leaves, the possibility of extrusion patterns would remain open.

METHOD

Waxes used in the recrystallisation studies described in this section were isolated by the method described in Chapter 3 — 'Isolation of leaf waxes'.

Preliminary investigations showed that an average of 2 mg/cm^2 of recrystallised wax gave sufficient wax to replicate and examine with the electron microscope. The respective waxes were redissolved in chloroform and a standard volume of 3 ml used in each case.

The recrystallisation conditions used were:

(i) 15 °C — uncovered vessel in still air conditions;

(ii) 36 °C — uncovered vessel in still air conditions;

(iii) 15 °C — covered vessel in still air conditions;

(iv) 36 °C — covered vessel in still air conditions.

(Where vessels were covered, the extent of covering was determined so that the evaporative rate of (iv) equalled that of (i).)

(v) ambient room conditions with a gentle cool air stream;

(vi) ambient room conditions with a gentle hot air stream, this being 38 °C at the wax surface (wax melting point 68 °C).

The substrate for the waxes was prepared by flooding 4 cm shallow flat-bottomed Petri dishes internally with 0.5% parlodion in amyl acetate. These were drained immediately and left to dry in a dust-free atmosphere overnight.

Representative wax solutions were poured into the dishes and these in turn placed under the various evaporative conditions mentioned above.

After recrystallisation was complete, the wax deposited on the parlodion film was shadowed and carbon-coated in the same manner as previously described for the preparation of leaf replicas (Chapter 1).

The carbon film was scored into small areas approximately the size of a grid and the Petri dish filled with amyl acetate.

The carbon replicas generally floated free of the wax-parlodion film substrate within a few minutes. They were subsequently transferred to a chloroform/acetone solution (70/30 v/v) overnight to dissolve any adhering wax or parlodion.

The replicas were finally picked up on uncoated 300-mesh grids, dried and examined directly in the electron microscope.

Where the recrystallized wax deposited as structures too large to replicate satisfactorily, it was examined directly by light microscopy

using a macro-lens system. Results were recorded with a Leitz-Aristophot camera attachment using 4" × 5" Kodak EKTAPAN film.

RESULTS

For ease of comparison, each plate depicting recrystallised wax has, as an inset, a micrograph of the *in vivo* wax pattern from the appropriate temperature.

Plate 4.1 a & b illustrates the structure of low and high temperature waxes after rapid recrystallisation in a 15 °C environment. Under these conditions both waxes recrystallise as globular structures. The low temperature wax formed globules measuring up to 0.5 mm in height while the high temperature wax globules measured less than 0.1 mm. Very little amorphous wax was observed between the large individual wax structures.

Plate 4.2 a & b illustrates the pattern of low and high temperature wax when recrystallised slowly at 15 °C. As in the rapid recrystallisation, here again both waxes formed large individual structures. The low temperature wax produced structures up to 2 mm in height under the slow evaporation conditions but otherwise it closely resembled that produced by rapid evaporation conditions.

On the other hand, the high temperature wax more closely resembled the *in vivo* wax pattern consisting as it did of large plate-like masses. This was in contrast to the crystallisation behaviour of the same wax under rapid evaporation conditions where, as noted above, the structure of wax deposited from solution was quite globular with no tendency to form plates.

Plate 4.3 a & b illustrates the pattern of low and high temperature waxes when rapidly recrystallised in a 36 °C environment. The low temperature wax under these conditions was found to produce poorly developed plates exhibiting a flat fused branching pattern, bearing little similarity to the *in vivo* pattern of either type of wax. The high temperature wax on the other hand deposited more frequently as plates having little in the way of secondary branching. These plates were similar in structure to the central fused areas of wax plates observed in high temperature wax structures *in vivo*.

Plate 4.4 a & b illustrates the patterns of low and high temperature waxes when slowly recrystallised in a 36 °C environment. Once again, the

low temperature wax was observed to have a poorly developed recrystallisation pattern. The wax deposited as largely amorphous structures similar to those produced at 15 °C (Plates 4.1a and 4.2a) but of a considerably smaller magnitude. Structural details could not be ascertained by light microscopy, but in the electron microscope small rod-shaped structures were evident around the perimeter of each wax mass. These bore some resemblance to the *in vivo* wax pattern. There was no tendency of this low temperature wax for form wax plates.

By contrast, the high temperature wax exhibited well-developed, narrow, spreading plates which resembled the *in vivo* high temperature wax, especially in the formation of complex narrow overlying branches arising from a fused central wax mass.

Plate 4.5 a & b illustrates the pattern of a low and high temperature wax when recrystallised under a hot air stream in otherwise ambient room conditions. The pattern produced under these conditions by the low temperature wax was structurally almost identical to its pattern *in vivo* although the wax rods formed were smaller. Some plate wax was also evident but this was a minor structural component.

The high temperature wax also produced, under these conditions, a pattern resembling its *in vivo* pattern. Recrystallised wax took the form of overlying wax platelets, while the presence of wax rods was only rarely observed.

Plate 4.6 a,b & c illustrates the close correspondence between rod structures in low temperature wax recrystallised under a hot (38 °C) air stream and their *in vivo* counterparts. For instance, no essential differences between the two can be seen with regard to the concentric ring structures along the length of the rods.

Plate 4.7 a & b illustrates the pattern of a low and high temperature wax when recrystallised under a stream of cool air in ambient room conditions. The two types of wax again exhibit differences in structure, where the low temperature wax tended to form rods or very narrow plates while the high temperature wax formed small broad plates.

The wax pattern for both waxes was apparently established within a matter of seconds since a sheet of recrystallised wax was observed to form on the solvent surface during the solvent evaporation.

Plate 4.8a illustrates the leaf surface of an exceedingly small apical leaf from a 15/10 °C grown plant. The surface structure comprised an amorphous wax layer with very little structural wax. The ease with which the replicas could be stripped from such surfaces was an indication that wax was present in appreciable quantities. The virtually structureless appearance of the wax on the surface of these very young leaves was in sharp contrast with the highly structured wax found on a mature leaf (Plate 4.8b).

DISCUSSION

The results showed consistent differences between the high and low temperature waxes when recrystallised under a range of conditions. In the case of recrystallisation at low temperatures, neither wax bore much resemblance to its form *in vivo* though differences in size of wax structures were found between the two. At high temperatures, the wax types recrystallised in patterns quite similar to their natural appearances. This result suggested that while the small chemical differences between the two waxes resulted in consistently discrepant crystallisation behaviour, actual similarity to the *in vivo* condition is dependent on the rate of solvent dispersal. Certainly the solvent and the substrate used for the recrystallisation studies would be quite dissimilar to those found *in vivo*.

Further evidence for a close link between the ultimate wax structure and the rate of removal of solvent resulted from studies under hot and cool air streams (Plates 4.5 and 4.7). Differences in structure of deposited waxes could only have resulted from differential rates of evaporation of solvent.

One of the myths dispelled by this study relates to the 'growth ring' appearance of wax rods. This structure can not be due to any form of phasic growth resulting from uneven flow during wax secretion (Juniper, 1960; Hall, 1967a). Their appearance in recrystallised wax, completely removed from anything resembling leaf morphology, suggests rather that it is a natural form of the wax mixture when developed under particular evaporative conditions.

It seems apparent then, that the small differences in chemistry of 15 °C and 36 °C wax observed in Chapter 3 are nevertheless sufficient to

confer on each of them a distinctive behaviour when crystallised out of solution. The closeness of the resemblance of recrystallised wax to that found on the leaf seems to depend on the rate of dispersal of solvent, regardless of whether this has been achieved by varying temperature or rates of air flow or a combination of both. Since structures closely resembling those found *in vivo* may be formed by manipulation of physical factors, the involvement of channels and pores in the shaping of wax structures would seem to be very minor or perhaps non-existent.

Finally, replication studies of very young apical leaves would seem to suggest that formation of the structural wax, in response to the environmental conditions, occurs after the secretion of an amorphous primary wax layer. Formation of highly structured wax has never been observed on surfaces where wax did not already exist in some form.

Plates 4.1 - 4.8

The following plates illustrate the results for waxy *Brassica napus* plants or for wax derived from such plants.

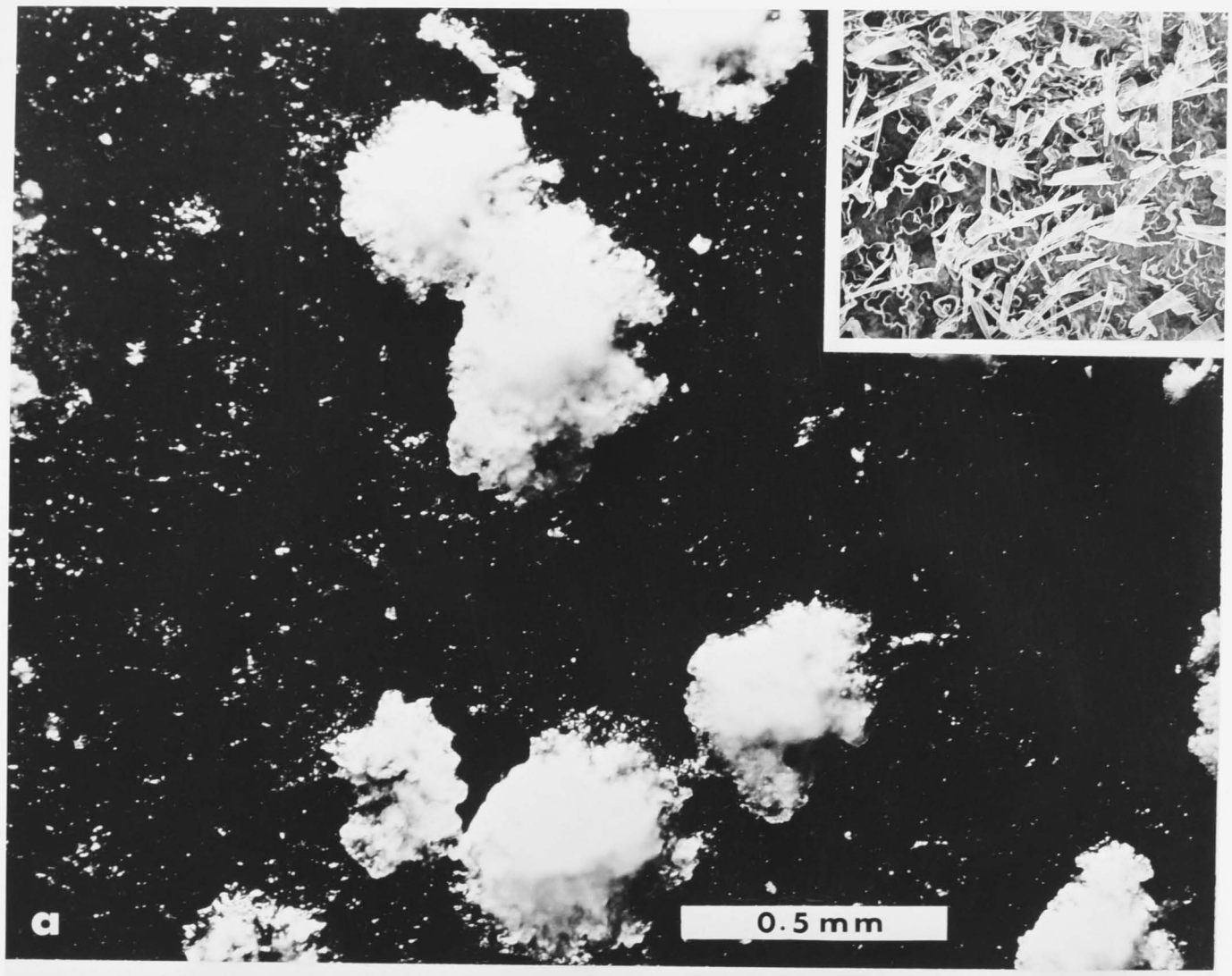
All E.M. wax recrystallisations and leaf surface preparations employed Pt/C shadowing and carbon replication.

The thin section micrograph was prepared from material fixed with glutaraldehyde/osmium tetroxide and stained with uranyl acetate and lead citrate.

Where appropriate, insets in the following micrographs illustrate the *in vivo* patterns of either 15/10 °C wax or 36/31 °C wax at low magnification. These are provided to aid in qualitative comparisons with the *in vitro* recrystallisation patterns of the same types of wax.

Dimension lines on micrographs represent 1 μm except where indicated otherwise.

Plate 4.1a,b: Light micrographs illustrating the typical wax patterns obtained when (a) 15/10 °C and (b) 36/31 °C grown waxes were recrystallised *rapidly* from chloroform solution at 15 °C.


a
0.5 mm

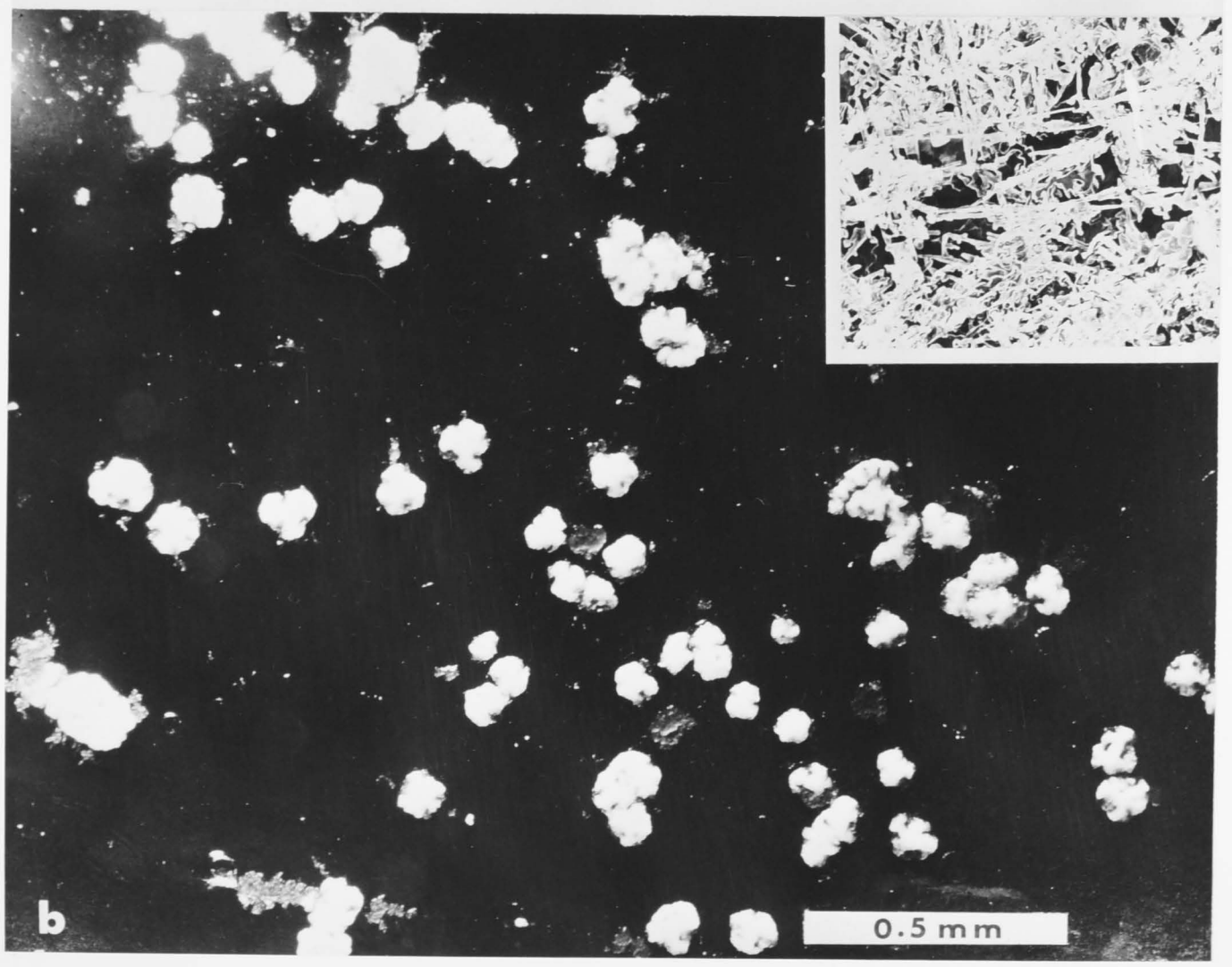
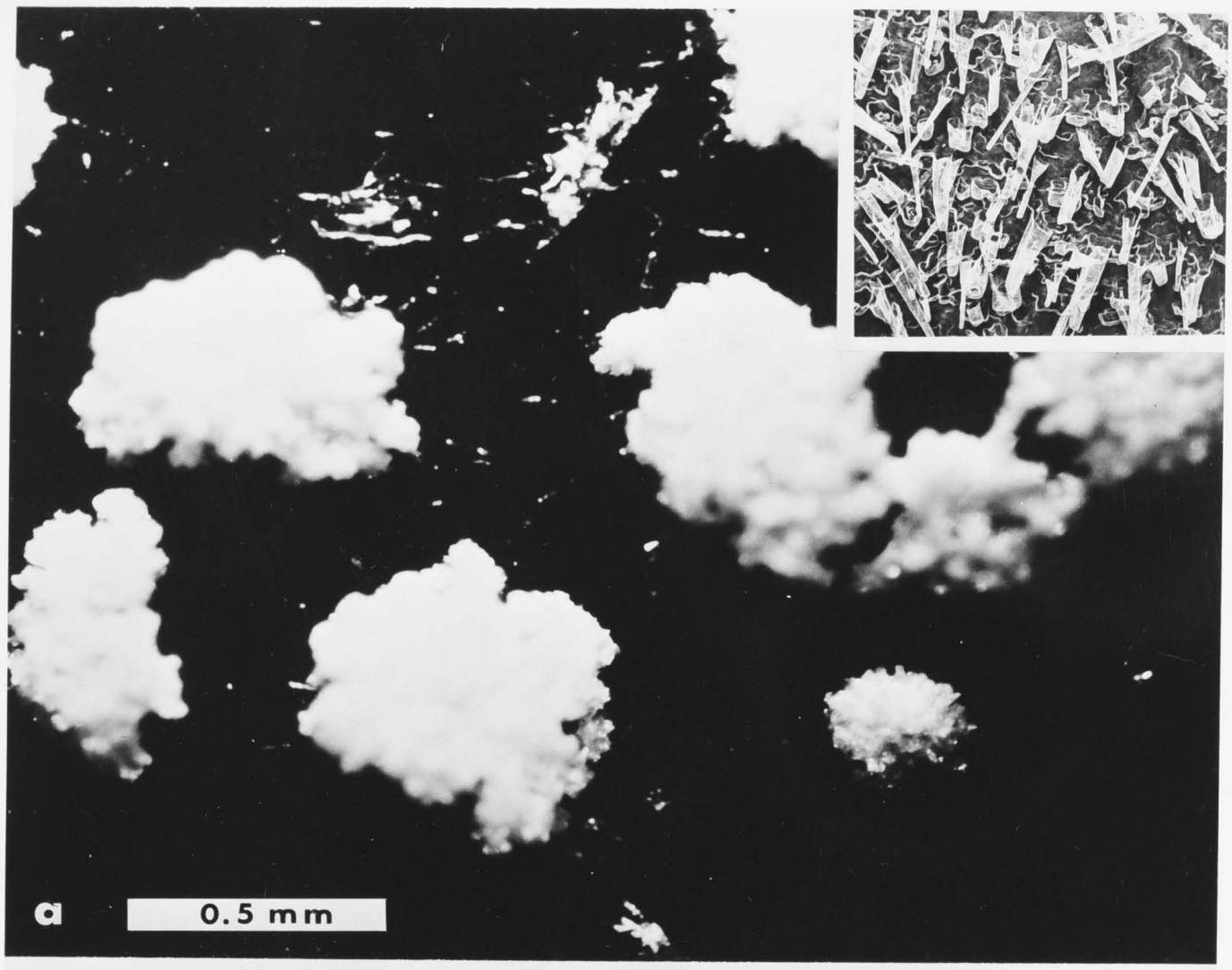

b
0.5 mm

Plate 4.2a,b: Light micrographs illustrating the typical wax pattern obtained when (a) 15/10 °C and (b) 36/31 °C grown waxes were recrystallised *slowly* from chloroform solution at 15 °C.


a
0.5 mm

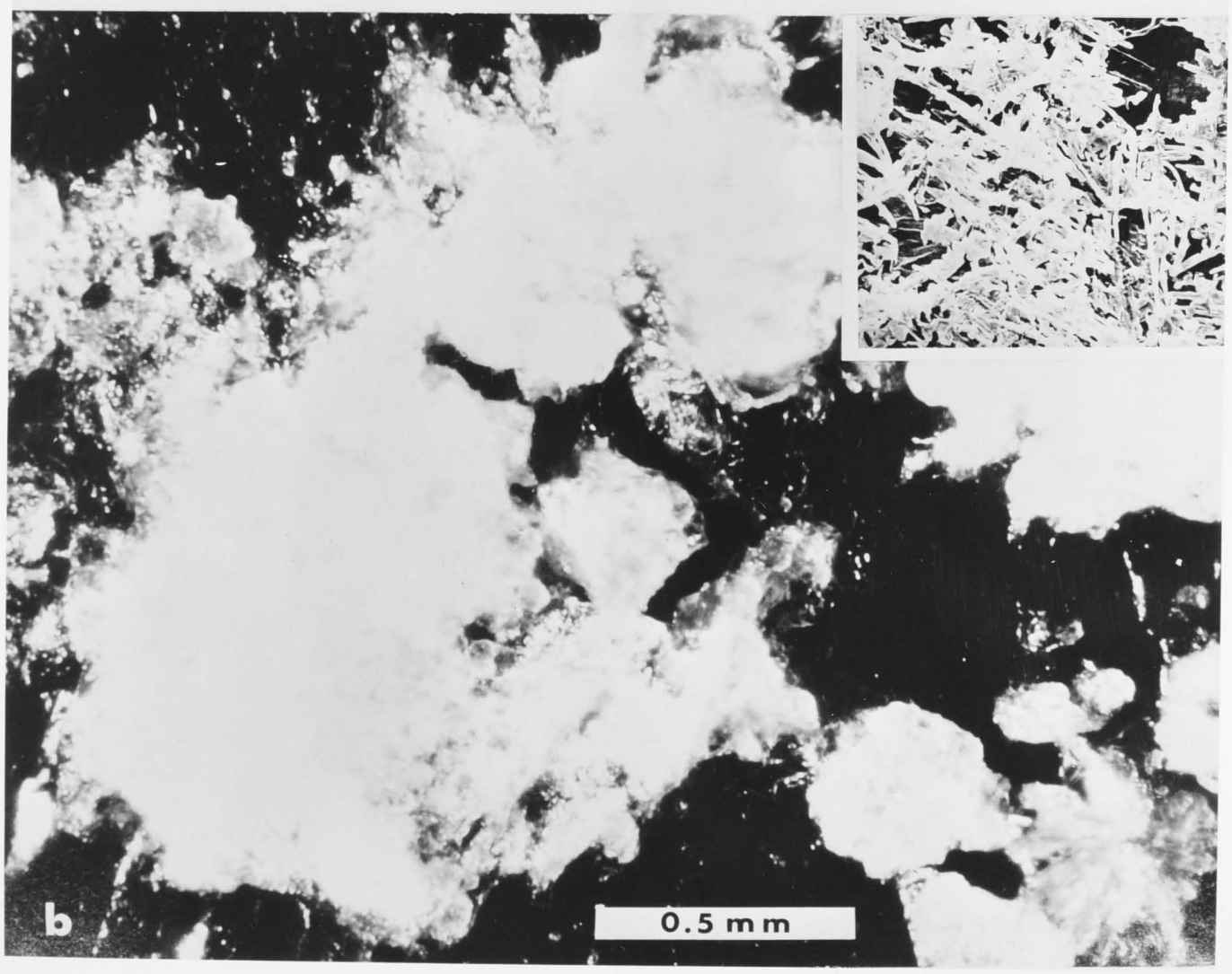
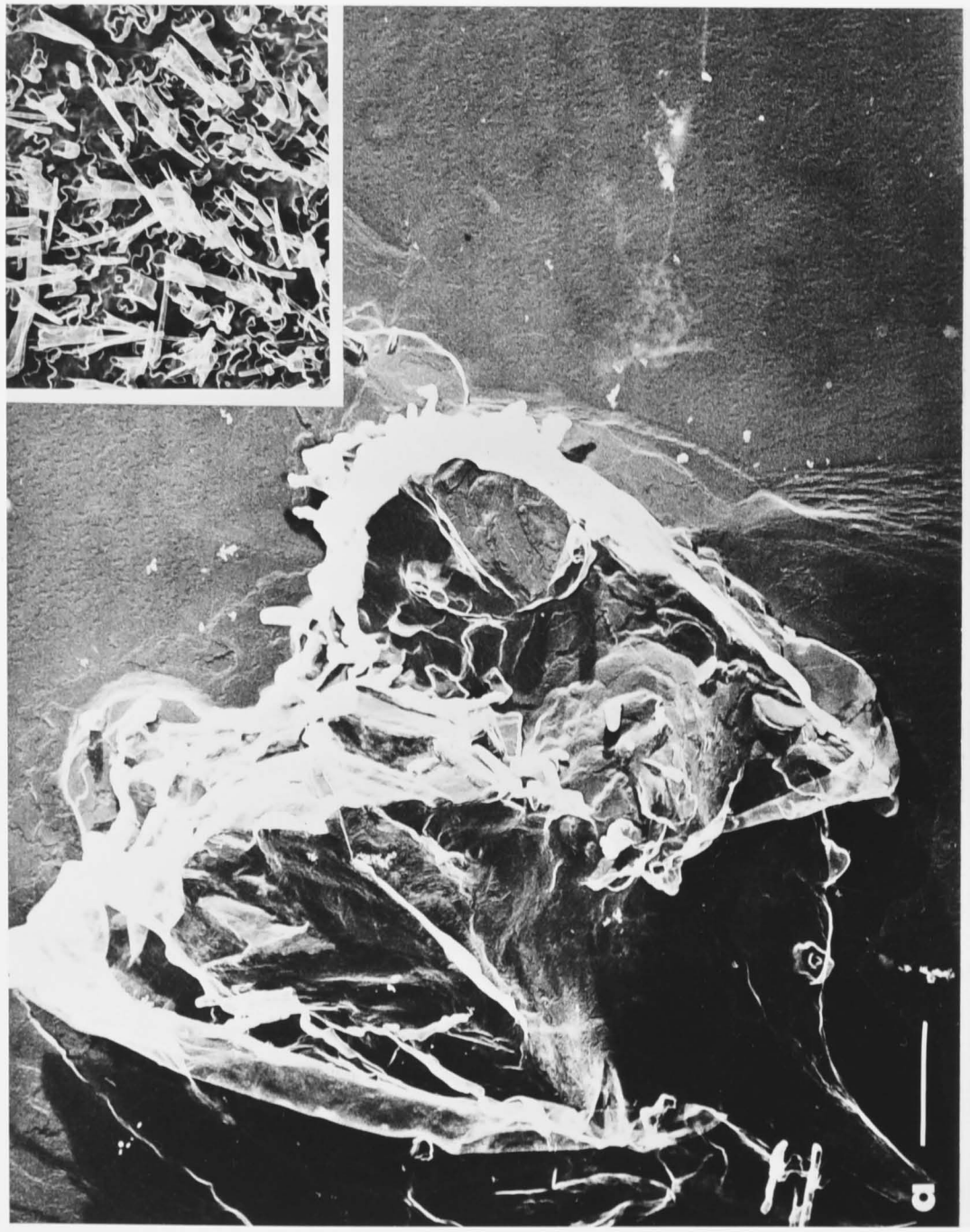

b
0.5 mm

Plate 4.3a,b: Electron micrographs illustrating the typical wax pattern obtained when (a) 15/10 °C and (b) 36/31 °C grown waxes were recrystallised *rapidly* from chloroform solution at 36 °C.

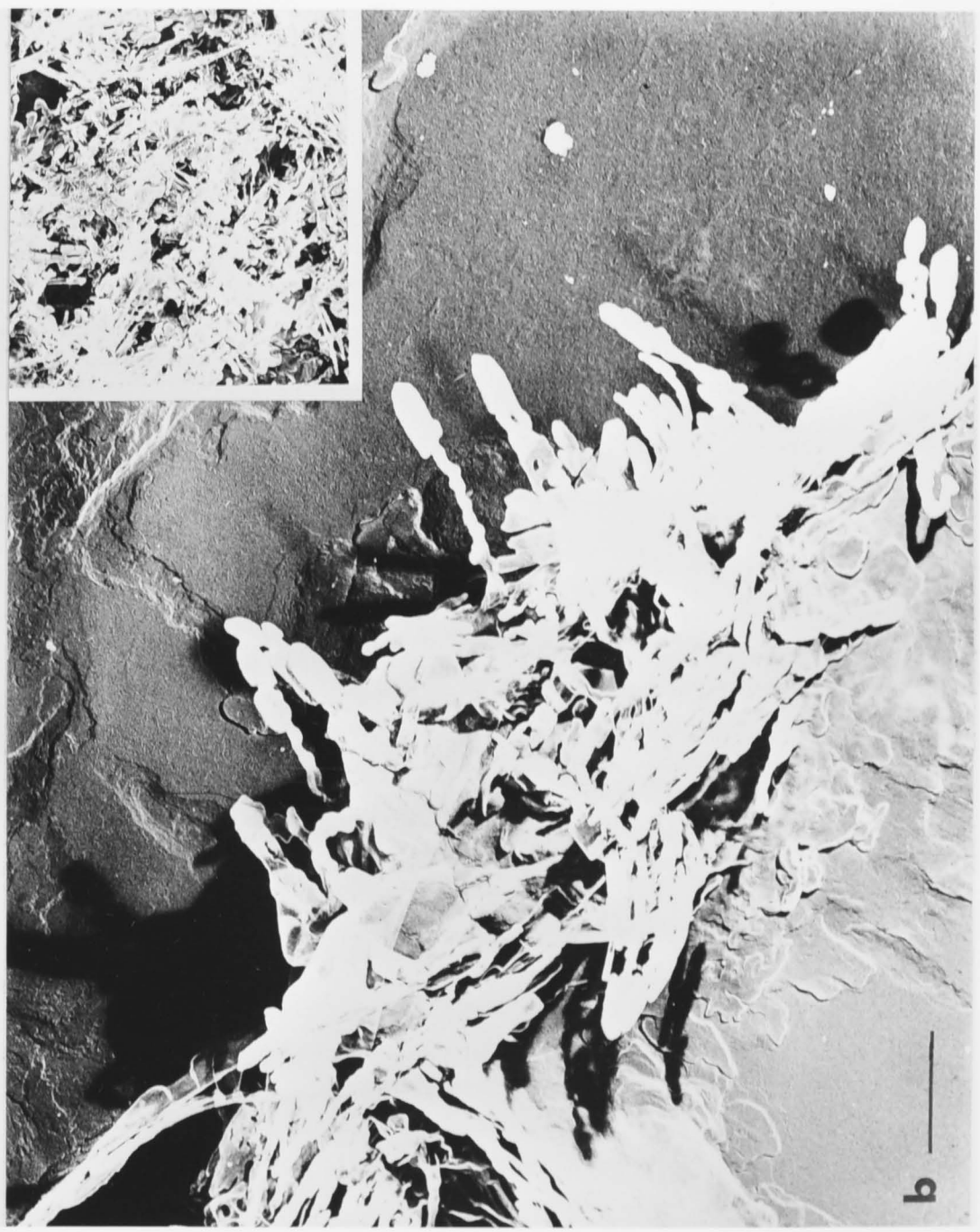

a


b

Plate 4.4a,b: Electron micrographs illustrating the typical wax pattern obtained when (a) 15/10 °C and (b) 36/31 °C grown waxes were recrystallised *slowly* from chloroform solution at 36 °C.



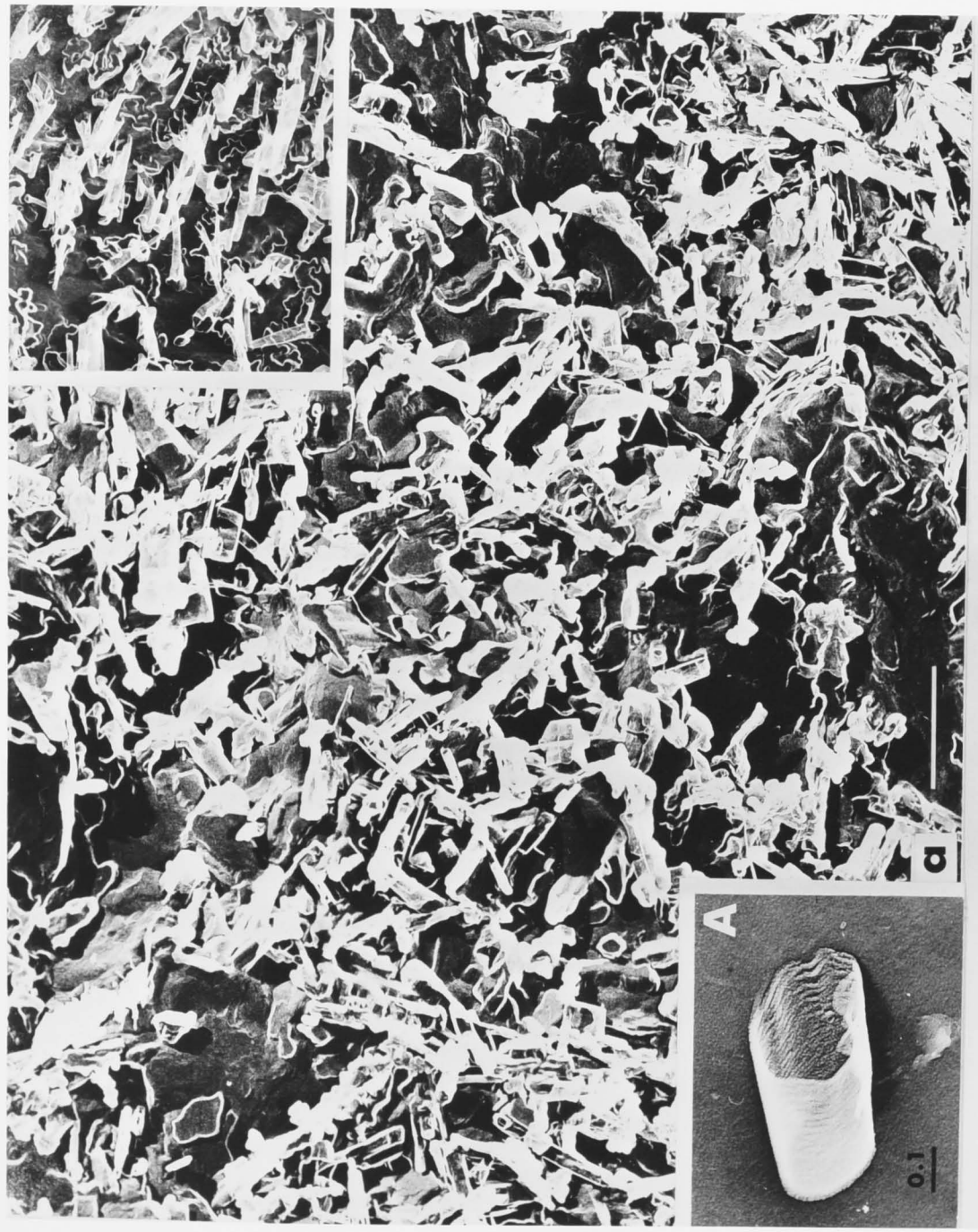
a



b

Plate 4.5a,b: Electron micrographs illustrating the typical wax pattern obtained when (a) 15/10 °C and (b) 36/31 °C grown waxes were recrystallised from chloroform solution under a hot fan (see text).

Inset A illustrates the concentric ring structure of the 15/10 °C wax under this evaporative condition.


a
A
0.1

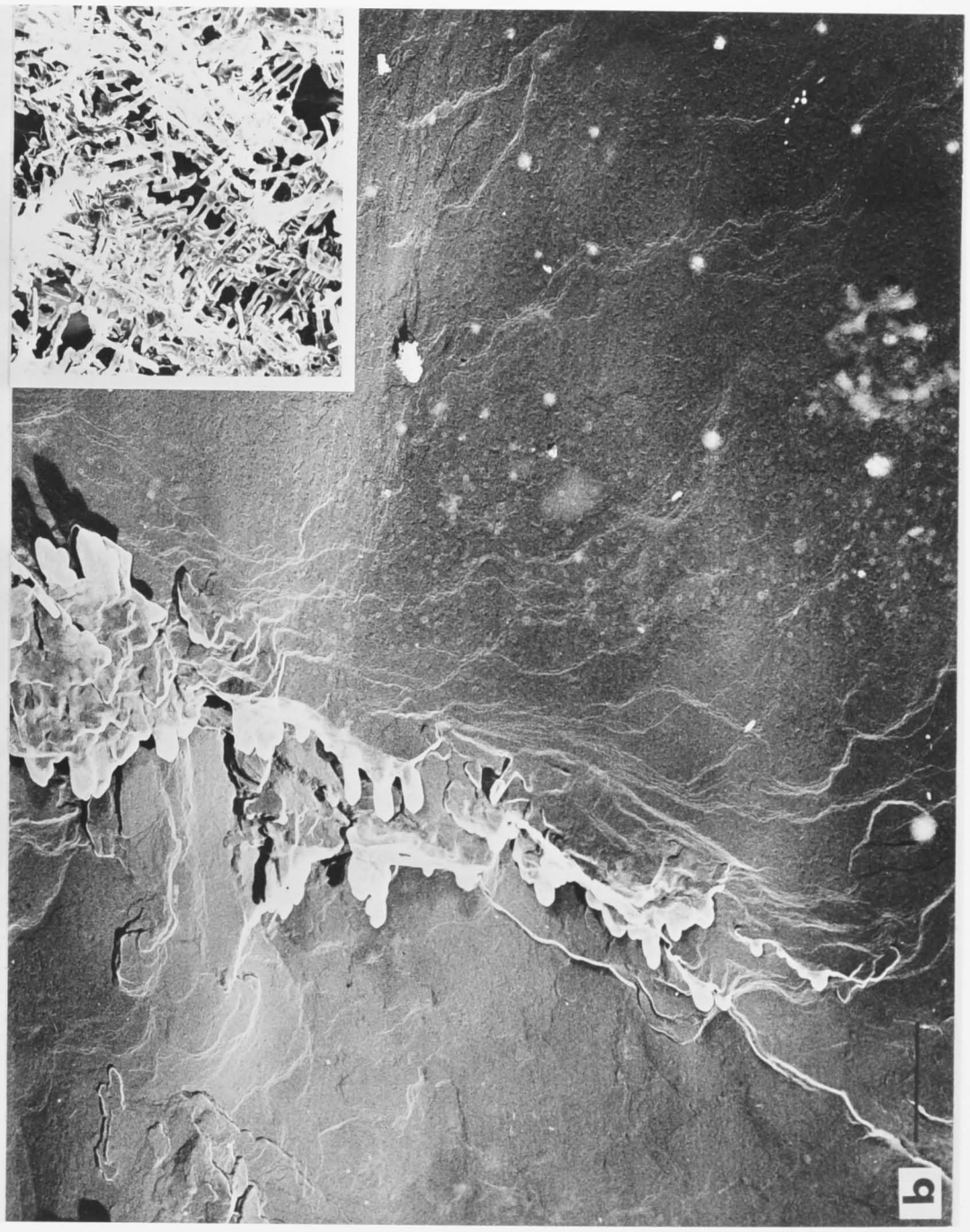

b

Plate 4.6: Electron micrographs illustrating the typical concentric ring structure of wax rods.

(a) *in vitro* wax structure of 15/10 °C grown wax recrystallised from chloroform solution (refer Plate 4.5a).

(b) *in vivo* 15/10 °C wax rods (C-replica preparation).

(c) *in vivo* 15/10 °C wax rods (thin section preparation).

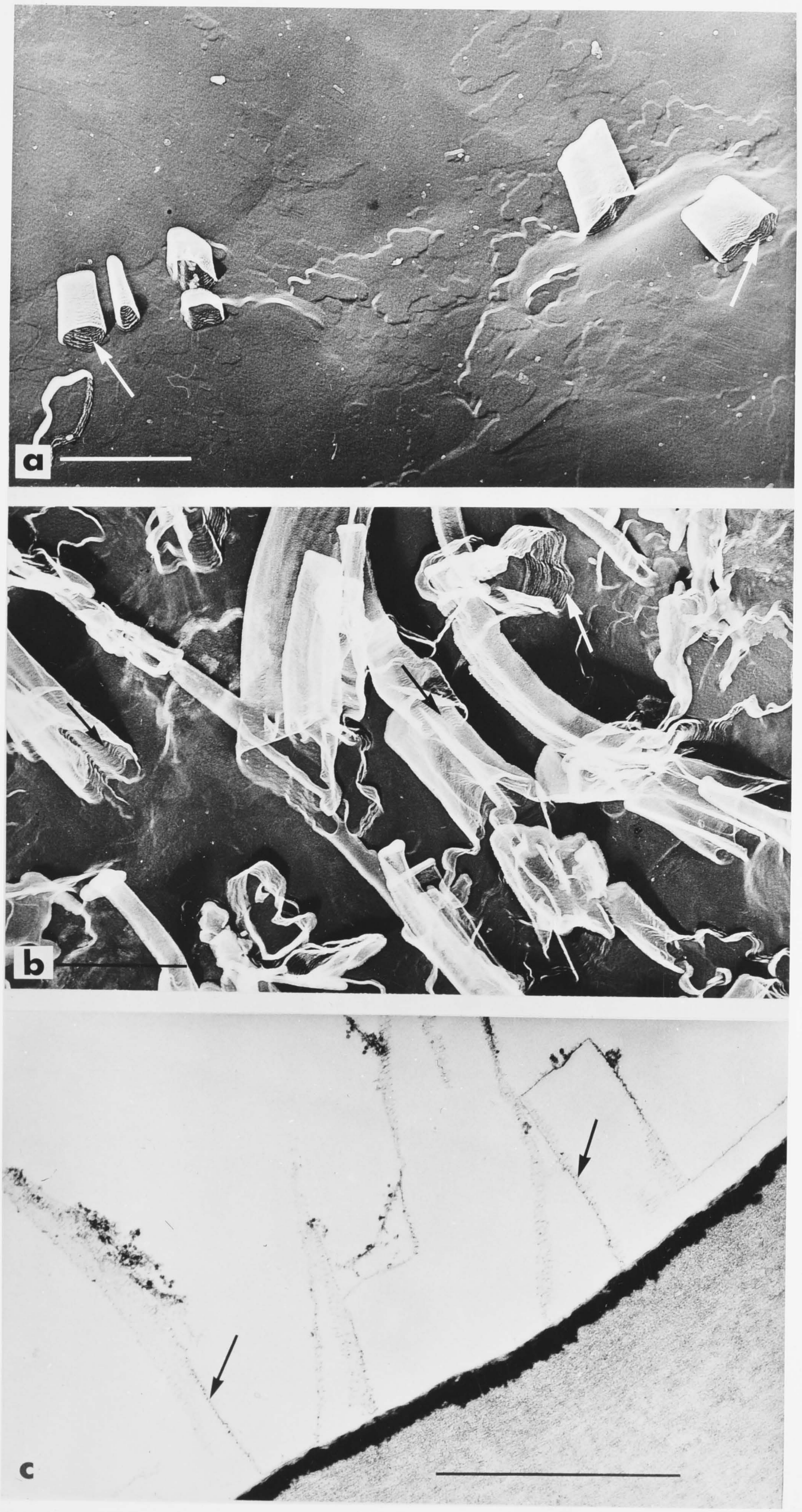

a
b
c

Plate 4.7a,b: Electron micrographs illustrating the typical wax pattern obtained when (a) 15/10 °C and (b) 36/31 °C grown waxes were recrystallised from chloroform solution under a cool fan (see text).

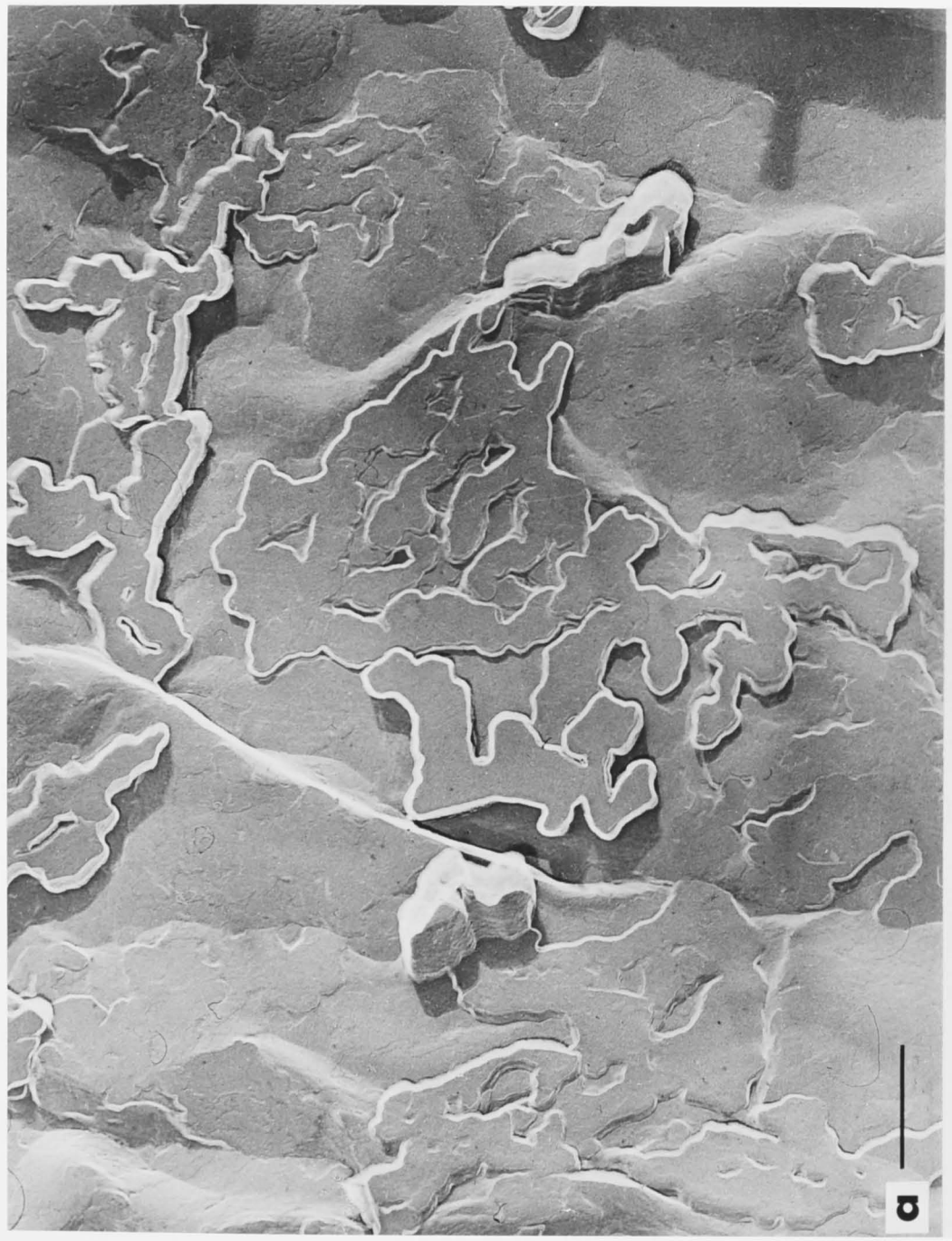

a


b

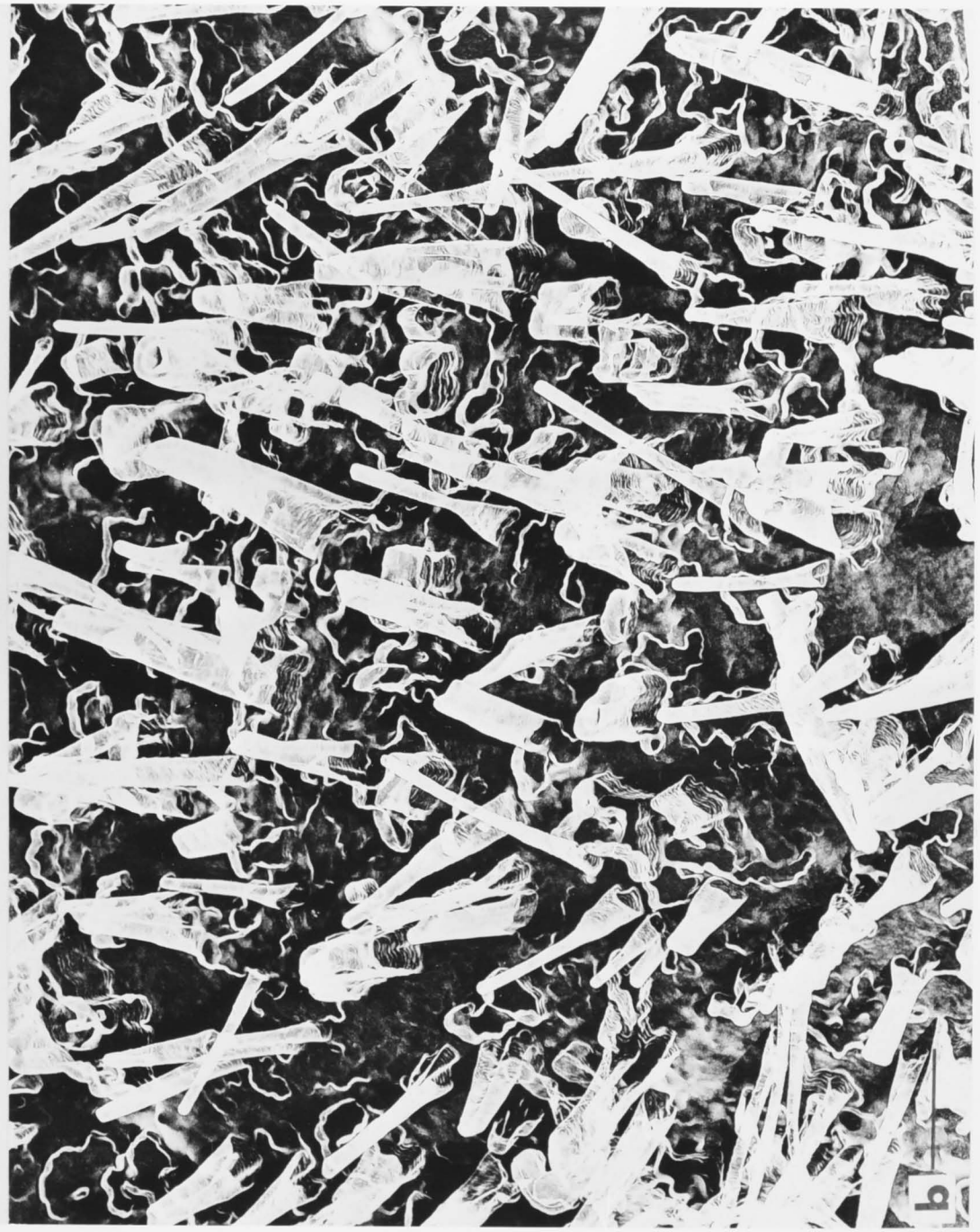
Plate 4.8: Electron micrographs illustrating the wax surface of

(a) a young apical leaf for a 15/10 °C grown plant.

(b) an expanded leaf for a 15/10 °C grown plant.



a



b

CHAPTER 5

CHAPTER 5

INCORPORATION OF ^{14}C-PALMITATE INTO EPIDERMAL CELLS AND SURFACE WAXES

INTRODUCTION

Radioactive fatty acids have been used extensively in characterising the biosynthetic pathways of plant waxes (e.g. Kolattukudy, 1970b; Macey, 1970; Macey & Barber, 1970a,b). Leaves have been found to assimilate ^{14}C-acetate into $C_{16} - C_{24}$ fatty acids (Macey & Stumpf, 1968) and into wax components, although the longer chain length compounds were found to be more efficiently incorporated (Kolattukudy, 1965).

Kolattukudy (1965) reported that administered palmitic acid was so rapidly incorporated into *Brassica* leaves that the leaf tip became labelled within five minutes of incubating the petiole in the labelled substrate. Incorporation of administered stearic acid into the wax was as high as 50% in four hours (Kolattukudy, 1966). Macey (1970) also noted very efficient uptake of palmitate into *Brassica* leaves.

1-^{14}C-palmitic acid was selected as a wax precursor in the present study because of its use in this role by previous workers, its high specific activity and its availability.

It has been the practice of some workers (e.g. Kolattukudy, 1966) to use non-ionic detergents, such as Tween-20, in making up solutions of long chain fatty acids. While the author could find no detectable effect due to detergent on the fine structure of the leaf cells, it was considered desirable to avoid the use of detergents in uptake experiments if at all possible. A means of complexing the palmitic acid to render it soluble in buffer solution was found and this is detailed in the methods section of this chapter.

Recent advances in cytology and cytochemistry have allowed, in some measure, correlations to be made between sites of biosynthesis and other specific enzyme reactions (using radioisotopes) and the fine structure of the cell. Such correlations have involved autoradiography since the nuclear emulsion is the only detector for radioisotopes with sufficient

powers of resolution to allow localisation at the level of individual cell organelles.

The use of radioactive tracer localisation with electron microscopy has been shown to have many associated problems however. The major difficulty in the autoradiographic study of lipid compounds has always been the problem of retention of label during fixation, dehydration and embedding procedures. While osmium tetroxide may be used to fix most unsaturated lipids, there has been no satisfactory means of fixing saturated lipids (Riemersma, 1963). Lipids are preserved to markedly different degrees by the various fixation and embedding procedures currently in use, but in no case are they preserved entirely (Idelman, 1965; Korn & Weisman, 1966; Casley-Smith & Day, 1966; Casley-Smith, 1967; Ashworth *et al.*, 1966; Morgan & Huber, 1967; Stein & Stein, 1968; Cope & Williams, 1968; Saunders *et al.*, 1968, Buschmann & Taylor, 1968; and Dermer, 1968).

So far, glutaraldehyde-osmium tetroxide fixation has been found to give the most satisfactory retention of lipids while at the same time giving the clearest morphological preservation (Idelman, 1965; Casley-Smith, 1963; Morgan & Huber, 1967; and Cope & Williams, 1968).

Loss of lipid during dehydration of tissue has been examined by Cope & Williams (1968). For the present study, several different dehydration procedures were examined, details of which appear in Appendix II.

Various embedding media have also been investigated with regard to lipid retention. Epoxy resins appear to retain as much lipid as any other media (Cope & Williams, 1968) including methacrylate at low temperature which conserved lipid no better than did conventional Araldite media.

Besides the problem of retention of lipid, various sectioning difficulties applicable to autoradiography have also been discussed by Williams & Meek (1966), while the use of nuclear emulsions and their method of application to the labelled sections are outlined by Caro & van Tubergen (1962), Granboulan (1965), Salpeter & Bachmann (1964), and Heremans (1971).

Resolution in EM autoradiography has been extensively studied by Caro (1962), Pelc (1963), and Bachmann & Salpeter (1965). The autoradiography of soluble compounds in relation to resolving power,

sensitivity and latent image fading has been examined by Appleton (1966, 1969).

Finally, many techniques, precautions and limitations of EM autoradiography have been recently outlined and discussed in an excellent review by Rogers (1971).

Among the numerous autoradiographic studies made in recent years, none to date has been specifically directed at incorporation of labelled wax precursors in order to localise wax synthesis at any particular site within the cell.

With little doubt the solubility of the components involved has deterred many workers from this field of investigation. Despite the fact that no truly satisfactory tissue preparation method exists, some lipid autoradiographic results at the electron microscope level have been published (Wilske & Ross, 1965; Jersild, 1966; Stein & Stein, 1966a,b, 1967; Williams & Carr, 1968).

In a comprehensive autoradiographic study, the results should be extensively analysed statistically as detailed by Williams (1969) and Rogers (1971). In a lipid study, however, since one is not certain how much differential extraction or translocation of label has occurred, extensive analysis and detailed investigation of such experiments is, in many cases, probably not justified.

The study outlined here was only moderately successful and the author was at all times aware of extraction and possible translocation problems. Nevertheless, because of the importance of this class of compound, even a moderate success was considered worth reporting and the study may serve as a guide to others.

METHODS

Experimental Standards

Genetically waxy and non-waxy (gl_3) leaves from plants grown at 24/19 °C were used for all radioactive experiments. A standard light intensity of 700 foot candles, achieved with tungsten lights was used.

Preparation of Radioactive Palmitate

A solution of radioactive palmitic acid (Calbiochem, 98% purity, specific activity 20 mc/mm) was prepared by dissolving 1-^{14}C-palmitic

acid in ether and evaporating the latter under a stream of nitrogen gas.

Bovine serum albumin in 0.025 M sodium phosphate buffer (pH 7.2) was added to the radioactive fatty acid to obtain a molar ratio of fatty acid to albumin of 5:1. The solution was sonicated for two minutes, its final concentration being 2.5 mM (Kendall, 1941; Goodman, 1957; Björntorp, 1968).

Incorporation of Palmitate and Wax Removal

The incorporation rate of ^{14}C-palmitic acid into leaf surface wax was monitored by counting the radioactivity in the wax isolated from a leaf after an uptake period. Leaves with laminae approximately 12 cm^2 in area having petioles approximately 1 cm long were kept in the dark with the petioles in water for two hours, during which time they became fully turgid. The petioles were then transferred to the labelled substrate to a depth of 2 mm. After various times of incubation, the lamina surfaces were washed in a wax solvent. Extreme care was taken to prevent contamination of the solvent from the petiole portion of the leaf.

While leaching of internal lipids during the solvent wash does not appear to be a significant problem (Kolattukudy, 1965), the author nevertheless was very conscious of this possibility. Bearing in mind the effect on fine structure of leaf cells of relatively short exposures to non-polar solvents (ref. Plates 3.1 and 3.2) and taking into account that 99% of the surface wax radioactivity is released within the first ten seconds of washing (Kolattukudy, 1965), the author standardised on washing labelled leaf waxes in n-hexane for eight seconds. All structural wax was removed by this procedure (Plate 3.1a).

Leaf surface washes were carried out on waxy and non-waxy (gl_3) leaves after various uptake periods. The leaves were dipped in distilled water for eight seconds or alternatively dipped in 0.2% detergent solution (Tween-20) also for eight seconds. The leaves were subsequently dipped in n-hexane to remove the labelled wax.

All solutions were carefully evaporated to dryness, placed in counting vials and 1 ml of chloroform added. After five minutes mixing to ensure that all solid wax was dissolved, 13 ml of 0.5% P.P.O. (2,5-diphenyloxazole) in scintillation grade tuolene was added as a scintillant and stirred for one hour using a teflon coated magnetic flea.

Contents were counted for carbon-14 using a Beckman LS-100 scintillation counter. Quench was relatively constant and an external standard showed little variation within the range counted. Differences in counting efficiency were negligible.

Autoradiography of Whole Leaves

The distribution of ^{14}C-palmitic acid within the leaf lamina was determined by placing labelled leaves against a sheet of Ilford 75W Red Seal X-ray film. The leaves, adaxial surface toward the film, were placed between two glass sheets and exposed for four weeks at -25 °C followed by ten weeks at 5 °C.

The exposed film was developed in Phenisol X-ray developer (diluted 1:4) for four minutes, fixed, washed and dried.

Combined Electron Microscopy and Autoradiography

A disc 1 cm in diameter was punched out of a 24/19 °C grown leaf and a sharp razor blade used to cut 2 mm parallel strips approximately 6 mm long. The cut disc was incubated on a 1-^{14}C-palmitic acid solution (prepared as described above) for two hours under a light source measuring 700 foot candles.

After incubation, the leaf disc was rinsed briefly in 0.025 M sodium phosphate buffer and subsequently treated as for the study of leaf fine structure (Chapter 1). The dehydration media and procedure used in this study was determined on the basis of minimum extraction of incorporated 1-^{14}C-palmitic acid and good preservation of fine structure. Details of extraction of label using various dehydration media and procedures in the preparation of *Brassica napus* leaf tissue were obtained in a preliminary study and are presented in Appendix II.

Sections of silver-gold interference colour were mounted on coated gold 200-mesh grids and stained in uranyl acetate (saturated aqueous) and Reynolds lead citrate. Sections were then coated with carbon (approximately 60 nm) (Rogers, 1971).

Emulsion Coating

Two methods were used to coat Ilford L4 emulsion on to the specimens, these being discussed in detail together with their various advantages in Appendix III.

Exposure

The coated grids were stored in a 10 °C environment with a drying agent present. While Ilford L4 emulsion does not appear to be highly susceptible to loss of latent image (Salpeter & Bachmann, 1964), the specimens were kept in an atmosphere of oxygen-free nitrogen gas throughout the exposure period. Grids were removed and processed at three weekly intervals to establish the correct exposure time.

Photographic Procedure

Grids were developed on a drop of Kodak D-11 (diluted 1:1) at 20 °C for four minutes. Only development times in excess of seven minutes cause a noticeable increase in the background using Ilford L4 emulsion (Caro & van Tubergen, 1962).

The grids were subsequently rinsed in distilled water for thirty seconds, fixed for two minutes and finally washed for ten minutes. After drying the grids were examined in the electron microscope.

RESULTS

(i) The radioactivity counts for the incorporation of ^{14}C-palmitic acid into leaf surface wax as removed by polar and non-polar solvents are presented in Table 5.1.

(ii) The distribution pattern of ^{14}C-palmitic acid into excised leaves is illustrated in Plate 5.1 a & b for the normal waxy and non-waxy (gl_3) mutant leaves. Uptake of the ^{14}C-palmitic acid solution was found to occur via the vascular tissue of leaves. Initial distribution of the label within the leaves varies for the waxy and non-waxy plants due largely to what appears to be a venation pattern difference. Further, the transpiration system would appear to be the major pathway for the uptake, since incorporation into the lamina was still evident after steam killing a portion of the petiole (I.F. Wardlaw, personal communication). The label was found to be distributed throughout the lamina of both waxy and non-waxy leaves after an incubation period of five minutes.

(iii) The autoradiographs (Plates 5.2 and 5.3) showed localisation of ^{14}C-labelling by the presence of silver grains over the outer epidermal cell wall, protoplast and surface wax regions. This result is supported to some extent by the work of Kolattukudy (1968) who found that

isolated epidermal strips of *Senecio odoris* readily incorporated ^{14}C-acetate and metabolised it into surface wax components. Occasional labelling inside the vacuole and outside the immediate surface wax boundaries has been observed. This most likely represents a leaching of the label from specific sites during the final embedding and polymerisation (Cope & Williams, 1968).

A relatively large grain size resulted from using a chemical developer (2 - 3 times that of the original silver halide crystal). This disadvantage seemed to be outweighed by improved sensitivity and minimal silver grain displacement noted when physical developers were employed. Minimum silver grain displacement was an obvious consideration in a study such as this due to the soluble compounds involved. In spite of the large silver grain size, the more specifically labelled organelles of the cytoplasm appear to be the mitochondria, endoplasmic reticulum, Golgi bodies and certain cytoplasmic vesicles.

Table 5.1

Total number of carbon-14 counts obtained for various leaf surface washings after incorporation of 1-^{14}C-palmitate into waxy and non-waxy leaves for various incubation times.

	Incubation time (minutes)	Distilled water wash ^{14}C counts min^{-1}	0.2% Detergent wash ^{14}C counts min^{-1}	n-hexane wash ^{14}C counts min
waxy leaves	5	0	-	700
	20	0	-	1650
	5	-	200	200*
	20	-	300	293*
non-waxy gl_3 leaves	5	82	-	516
	20	120	-	1140
	5	-	138	202*
	20	-	320	410*

* The quantitative significance of these results is in doubt since a non-polar solvent was used on a leaf surface already wet with a polar solution, thereby making wax removal incomplete.

\- Indicate inapplicable steps.

DISCUSSION

Isolated waxy and non-waxy leaves of rape were found to metabolise incorporated ^{14}C-palmitic acid into leaf surface waxes in as little time as five minutes. Similar results have been reported in *Brassica oleracea* by Kolattukudy (1965). Incorporation times of less than five minutes, even reduced to two minutes, produced detectable amounts of radioactivity in the isolated leaf wax. These results were generally highly variable with their magnitude seemingly related to a physiological state of the leaf at the time of the incorporation.

Varying the ^{14}C-palmitate incorporation time for the waxy leaves showed no water soluble components to have been distributed in the upper wax layers of the leaf. The exceedingly water repellent nature of the waxy leaf surfaces concerned presumably prevented a gentle distilled water wash gaining access to any labelled water-soluble component in the substructural wax or in the substomatal cavities. A subsequent wax solvent wash readily removed the structural wax present on the leaf surface, including that wax metabolised during the incorporation period.

In contrast to the waxy leaves, the non-waxy labelled leaves showed a small but detectable removal of radioactive components when washed in distilled water. While the surface of the non-waxy leaves has essentially no structural wax (Plate 1.15c) when compared with a 24/19 °C waxy leaf (Plate 1.14a), sufficient wax is still present on the former to make the surface effectively non-wettable (contact angle measured at 94° ± 3°11' compared with a contact angle of 141° ± 1°27' for the waxy leaf; method of Fogg (1947)). The shorter chain length wax components (Macey & Barber, 1970b) involved would presumably largely account for the detectable counts obtained for the non-waxy leaves washed in distilled water. A subsequent solvent wash, as with the waxy leaves, was found to remove surface wax synthesised and transported during the incubation period.

The action of a weak detergent solution was to significantly reduce the water repellency of both waxy and non-waxy leaf surfaces. Considering that much of the leaf surfaces were wetted, it seems likely that labelled sites in the substomatal cavities and substructural wax regions may have been reached by the detergent solution. As with the other cases, a wax solvent wash was found to remove labelled wax;

however, due to the fact that a non-polar solution was used on a surface already largely wetted with a polar solution, not all the wax would have been removed.

The autoradiographic results presented in this study are similar to those of Stein & Stein (1966a,b) who studied the distribution of ^{14}C-palmitic acid in rat liver. The cytoplasm of *Brassica napus* epidermal cells have been shown in this study to have only small numbers of mitochondria and Golgi bodies, and sections lacking these organelles completely were not uncommon. In these experiments, the mitochondria and Golgi bodies, where present, showed definite signs of having incorporated some radioactivity, in the same way that labelled palmitate was found to be localised in rat liver mitochondria and Golgi bodies (Stein & Stein, 1966a,b).

Although mitochondria are capable of synthesising and oxidising long chain fatty acids (Martin & Stumpf, 1959; Stumpf, 1965; Wakil, 1961; Björntorp, 1968), the incorporation of label into mitochondria from exogenous ^{14}C-palmitate does not itself argue that mitochondria synthesise surface wax or their precursors. Mitochondrial labelling was also apparent within the palisade cells of the leaf. It has been suggested that Golgi bodies may be involved in lipid storage (Moor & Mühlethaler, 1963), while Hallam (1970b) has noted an apparent movement of Golgi bodies in association with cytoplasmic vesicles toward the plasmolemma. In the light of these observations, it is tempting to suggest the involvement of Golgi and cytoplasmic vesicles with storage or transport of wax precursors but this has not been clearly established.

In these experiments, the most consistently labelled cell organelle was the endoplasmic reticulum. All autoradiographs showed labelling of endoplasmic reticulum even where no other major cell organelle was present, implying that at least active incorporation of palmitic acid occurred. The tentative suggestion may be made that the endoplasmic reticulum provided a site for palmitate esterification. Whether the end products of such esterifications were the final wax components, or else some long chain intermediate, certainly could not be ascertained from this study. Most likely most of the labelled sites represent metabolised components of greater than C_{16} chain length since palmitate, being saturated, would not have been chemically fixed during the tissue

preparation and would thus have been subject to considerable leaching during the dehydration-embedding process.

The random distribution of silver grains across the cell wall from the plasmolemma into the surface wax regions might indicate a general movement of the incorporated label from the cytoplasm to the surface wax. Conclusions as to whether the label followed some fixed directional pathway across the cell wall could not be obtained from these autoradiographs. Loss of label by leaching during the dehydration and embedding process may have destroyed any such pattern if it did exist. Without further evidence one could only conclude from these investigations that surface wax components or some intermediate precursors move more or less randomly across the cell wall and cuticle before being deposited on the surface.

The use of fixed and embedded material for autoradiographic electron microscopy imposes serious limits on the application of the method, particularly in the study of lipids. The use of freezing ultramicrotomes to provide frozen sections for this kind of work may be successful. Preliminary reports of work with frozen sections have been promising (Appleton, 1969; Christensen, 1969; Hodson & Marshall, 1969), but the technical difficulties involved in adapting frozen sections to electron microscope autoradiography are immense.

Plates 5.1 - 5.3

The following plates illustrate the results for waxy *Brassica napus* plants, unless specifically stated otherwise.

Electron microscopic-autoradiographs were prepared by the incorporation of ^{14}C-palmitate into *Brassica napus* leaf tissue, followed by fixation with glutaraldehyde/osmium tetroxide and staining with uranyl acetate (saturated aqueous) and lead citrate. Ilford L4 nuclear emulsion was used for detecting the presence of radioactivity within the leaf section.

Dimension lines on micrographs represent 1 μm except where indicated otherwise.

Plate 5.1: Autoradiographs illustrating the distribution of ^{14}C-palmitate after 5 minutes incorporation in

(a) a waxy leaf.

(b) a non-waxy (gl_3) mutant leaf.


a 1.5 X

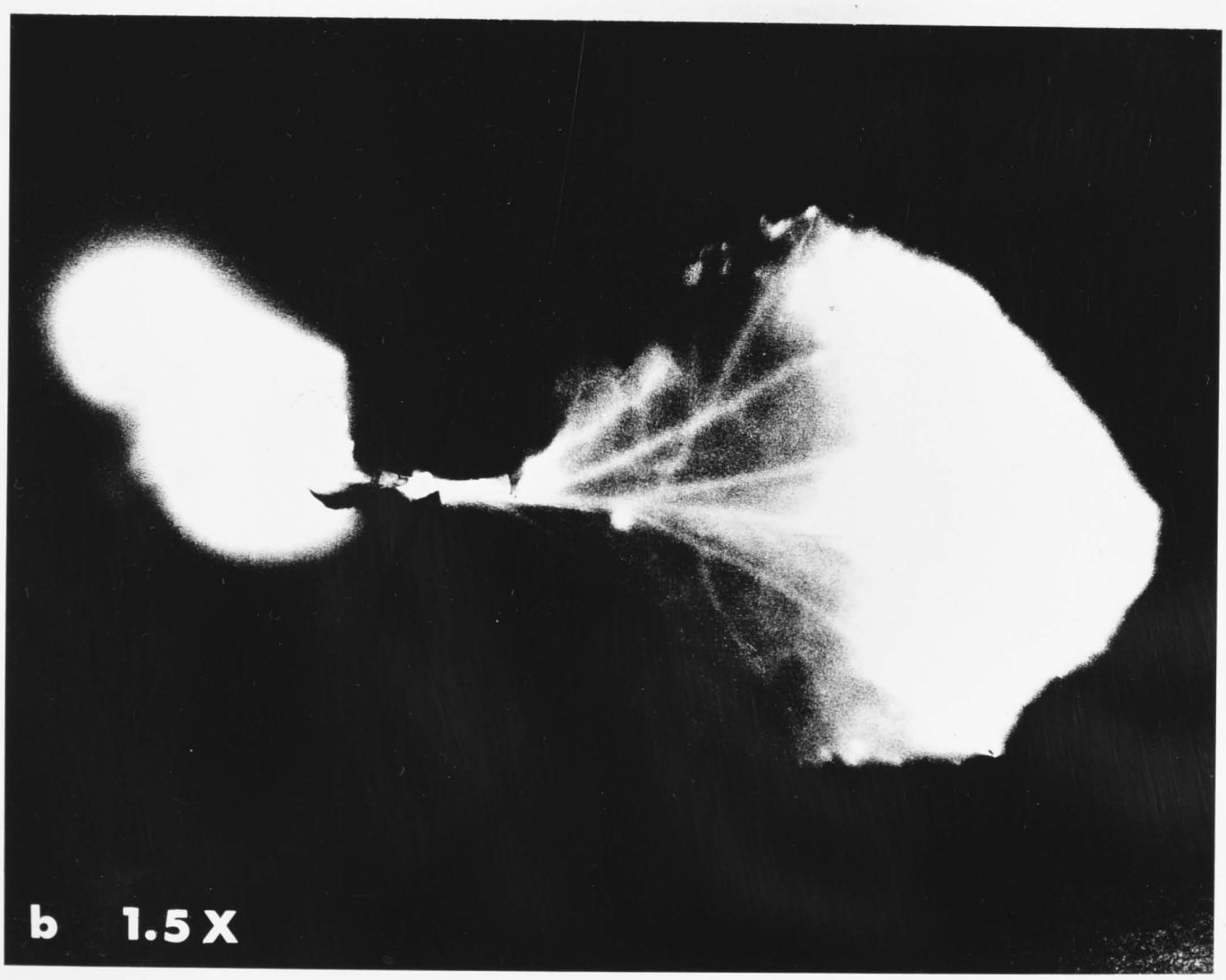

b 1.5 X

Plate 5.2a,b: Two E.M.-autoradiographs illustrating the localisation of silver grains over labelled areas of the outer tangential epidermal cell wall region after incorporation of ^{14}C-palmitate.


a

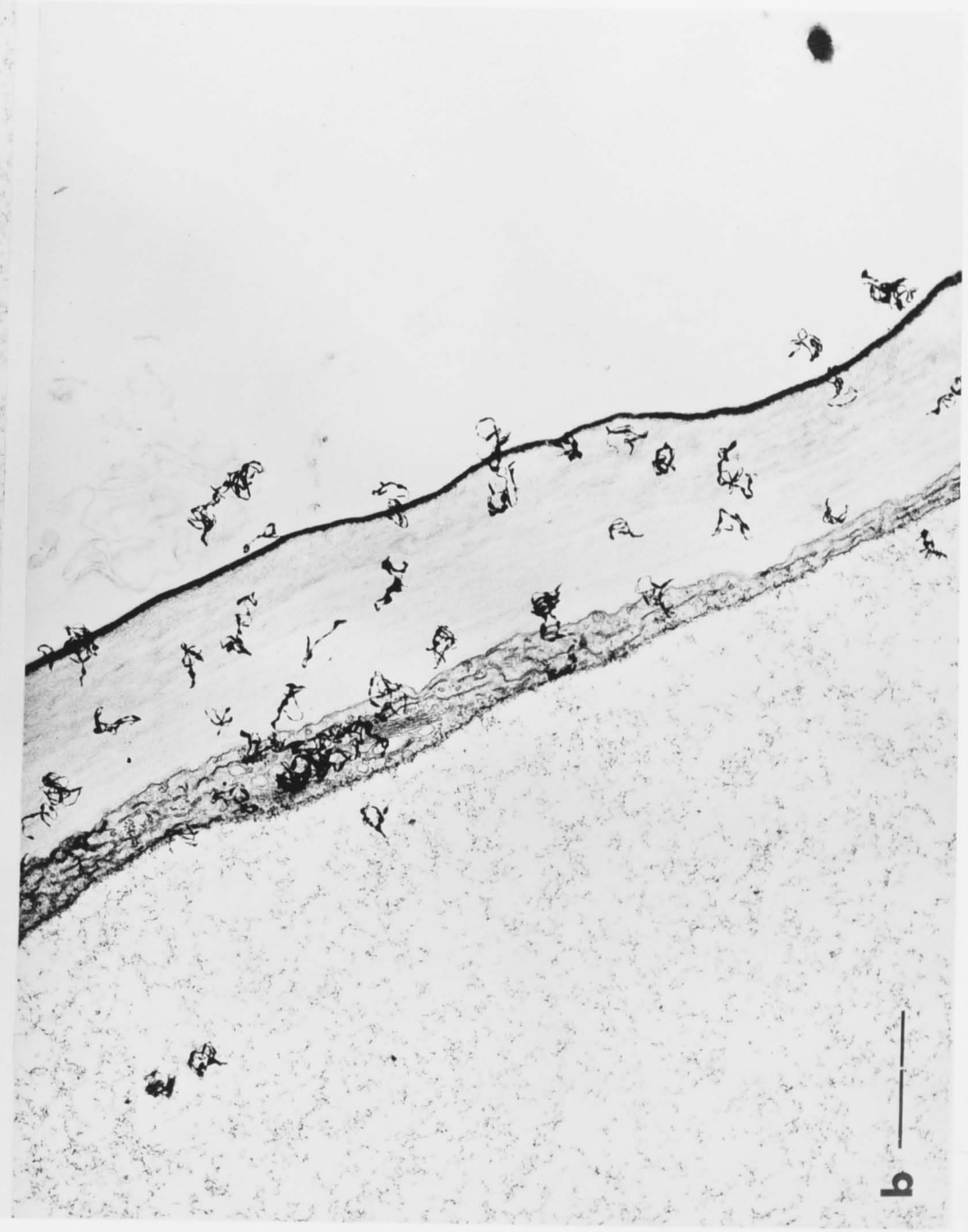
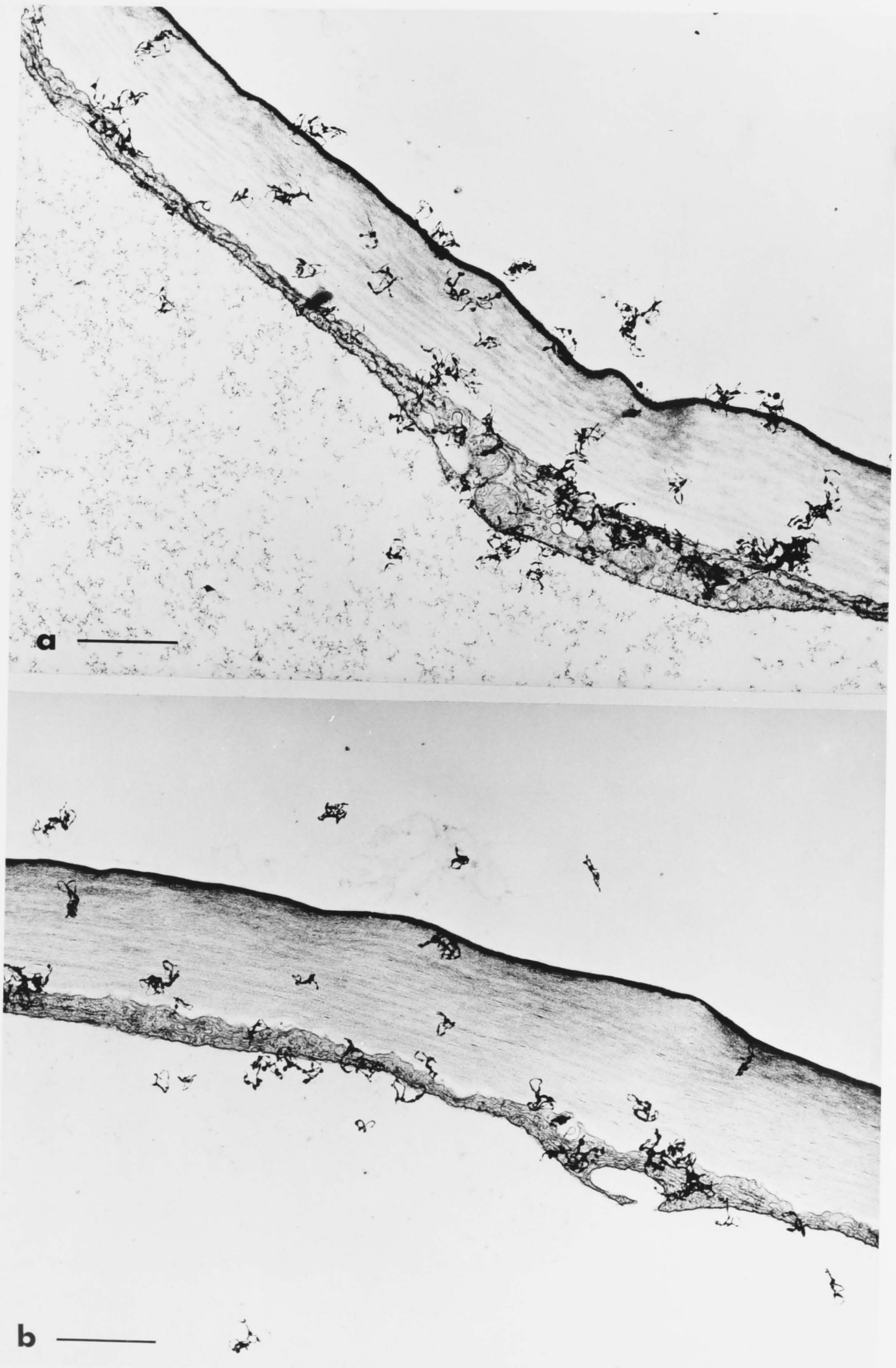

b

Plate 5.3a,b: Two E.M.-autoradiographs illustrating the localisation of silver grains over labelled areas of the outer tangential epidermal cell wall region after incorporation of ^{14}C-palmitate.


a
b

GENERAL CONCLUSIONS

Electron microscopic studies, using the techniques of conventional carbon replicas and scanning electron microscopy, have confirmed the variation in surface wax pattern for *Brassica napus* as achieved by a wider temperature range than previously reported. A marked transition from predominantly rod-type wax at low temperature to a predominantly branched platelet type (up to 36 °C) has been clearly established. Temperatures above this limit were impractical from the viewpoint of physiological breakdown.

Waxes from various temperature grown plants were removed and isolated by a procedure in which every precaution was taken to prevent *in vitro* transformations. The waxes on analysis by gas-liquid chromatography and mass spectrometry were shown to have small but consistent differences in the C_{29}, C_{31} hydrocarbons and the C_{29} symmetric ketone fractions. These studies also revealed the presence of ester compounds having chain lengths of up to C_{45}.

Fine structure studies of epidermal cell cytoplasm did not reveal any pronounced temperature effects able to be characterised cytologically. When the study was extended to wax mutant plants and plants treated with dewaxing herbicides such as T.C.A. and dalapon, again, little in the way of cytological discrepancies could be demonstrated as a result of the treatments concerned. These findings initiated an E.M. - autoradiographic study to determine the localisation site(s) of wax synthesis despite the difficulties of lipid retention and translocation of label inherent in such studies. The evidence gained implicated the endoplasmic reticulum and Golgi vesicles in the esterification and transportation of waxes, or wax precursors, respectively.

As to the pathway of wax from cells to leaf surface, an electron microscopic study involving serial sections of the outer epidermal walls and cuticle gave no suggestion of wax microchannels within the cell wall, though there was confirming evidence for microchannels and 'pores' in the cuticle itself. The 'pores' on the cuticular surface, by their size and distribution would seem to have no effect on the shape of the ultimate

wax deposits. Rather their rôle in the wax story seems to be simply that of outlets to the surface following the establishment and polymerisation of the basic leaf cuticle.

Recrystallisation studies on the high and low temperature grown wax confirmed that the shape of wax rods did not result from pore extrusions as has been frequently suggested in the literature. Indeed, the same 'growth ring' pattern on the surface of wax rods *in vivo* could be induced by certain physical conditions *in vitro*. It was concluded that the wax pattern produced *in vivo* on the surface of *Brassica* leaves was a result of both the chemical composition of the wax produced and the conditions themselves as they affect the rate of wax deposited from solution and thus the size and shape of the wax structures.

Further, it has been shown in a leaf still undergoing a wax movement phase that a transfer from an original low temperature environment to a high temperature environment resulted in a rapid transformation of existing low temperature wax, as well as the characteristic deposition of newly formed high temperature type wax. These changes have been found to be quite irreversible.

This study has, for *Brassica napus* at least, elucidated much of the speculation surrounding the synthesis of surface waxes, their transport to the leaf surface and their ultimate formation. Fatty acid wax precursors presumably are esterified in close association with the endoplasmic reticulum and are subsequently transported to extracellular regions by vesicles associated with Golgi bodies. Movement across the cell wall and cell wall - cuticle zone occurs by non-localised diffusion resulting from a concentration gradient, whereafter the wax moves via a random microchannel system ultimately reaching the surface by means of cuticular pores. The subsequent evaporation of the wax solvent, as yet unknown, results in a semi-crystalline wax layer, the final wax form being determined by an interaction between wax chemistry and environmental conditions.

APPENDIX I

LEAF THICKNESS MEASUREMENTS

The difficulty with the measuring of leaf thickness on leaves that are both 'soft' and have prominent veins on the abaxial surface was overcome by constructing the following apparatus.

On the jaws of a No. 1 metric micrometer, two 1 mm diameter contacts were glued, the contact on the fixed jaw being insulated from the remainder of the body. The contacts were wired up to a sensitive resistance meter such that there was zero resistance when the contacts were closed.

The leaf of a plant was inserted *in situ* between the open contacts and the moveable contact carefully closed until a circuit was formed as indicated by a registration of the meter. This reading was of the order of 10,000 ohms. A reading of considerably less than this figure was a good indication that the contacts has been closed too far causing local cell damage.

This method provided rapid and reproducible measures of leaf thickness without the necessity of excising the leaf concerned from the plant.

APPENDIX II

E.M. - AUTORADIOGRAPHY OF LIPIDS: LOSS OF LABEL RESULTING FROM VARIOUS DEHYDRATION PROCEDURES

In order to ascertain a dehydration procedure which would extract a minimum amount of radioactivity incorporated into leaf lipids, and would also maintain a good fine structure preservation, the following method was adopted.

Leaf tissue was labelled as described in Chapter 5, and processed through a glutaraldehyde/osmium tetroxide fixation procedure (Chapter 1, Methods).

A variety of dehydration media and dehydration times was subsequently used, and the various solutions were conserved and transferred to scintillation vials and evaporated to dryness.

At the completion of each dehydration run, the plant material remaining was combusted by a method used by Sands (1972), whereby all the solid material was converted to carbon dioxide. An absorbent of 1:1 2-phenylethylamine and anhydrous methanol was used to quantitatively absorb the carbon dioxide (Woeller, 1961).

Two ml of absorbent were added to counting vials and stirred for twenty-four hours using a teflon coated magnetic flea. Twelve ml of 0.5% P.P.O. (2,5-diphenyloxazole) in scintillation grade toluene was then added as a scintillant and stirred for five minutes.

The contents were counted for carbon-14 by the method described in Chapter 5.

Radioactivity (% of total counts) removed from *Brassica napus* leaf tissue by successive steps in four fixation-dehydration sequences, and final retention of radioactivity by the tissue.

Solution sequence	Dehydration Medium			
	Hexylene glycol	Ethanol	Acetone	Acetone
Fixatives* & washes	9.1	14.0	17.9	16.4
Dehydration† steps (% dehydrant)				
10	0.7(20)	-	-	-
20	1.5(20	5.3(20)	-	5.9(30)
25	-	-	5.6(10)	-
40	8.4(20)	8.0(20)	-	11.2(25)
50	-	-	22.9(10)	-
60	8.9(20)	12.8(20)	-	11.5(20)
75	-	-	17.9(10)	-
80	9.0(20)	9.0(20)	-	8.4(15)
100	11.1	6.7(20)	6.5(10)	4.7(15)
Propylene† oxide	-	6.6(20)	-	-
Residual tissue	51.3	37.6	29.2	41.9
Tissue§ preservation	Poor	Good	Fair	Good

* Glutaraldehyde/osmium tetroxide sequence for thin sections, Chapter 1, Methods.

† Figures in brackets represent time in each solution (minutes).

§ Ascertained on tissue taken through the same sequences after embedding and sectioning.

APPENDIX III

PROCEDURE FOR COATING NUCLEAR EMULSION ON ELECTRON MICROSCOPE SECTIONS

Two methods are described for the coating of Ilford L4 nuclear emulsion on to grids for use in EM autoradiography.

The emulsion was melted at approximately 45 °C in a water bath with distilled water (dilution 1:3). From initial investigations, this dilution was found to give a film consisting of a monolayer of closely packed silver halide crystals.

After gently stirring for 10 - 15 minutes, the diluted emulsion was poured into a Petri-dish and cooled. Caro & van Tubergen (1962) recommended cooling at 0 °C for some minutes, then allowing the emulsion to reach room temperature for half an hour. The author's experience has been that this preliminary cooling in an ice bath has little effect on the final properties of the emulsion film. What did aid gelling and prevent subsequent breakage of the film in the loop was to leave the emulsion standing at room temperature for 2 - 3 hours prior to use.

A platinum loop, 4 cm in diameter, was immersed in the emulsion and slowly withdrawn vertically, forming a film. Grids were supported on round cover slips which in turn were supported on rubber tower supports. The film held horizontally was brought down over the raised coverslip, thus contacting the grids as it broke from the loop.

As a control, some coated grids were examined with the electron-microscope to check the distribution of the silver halide crystals. Using this relatively simple technique the films formed were generally quite good. The distribution of the halide crystals, however, was almost always greater near the grid bars compared with that near the centre of grid openings. Another possible disadvantage of this method results from the emulsion film being applied while still labile. Some movements of halide crystals after coating does occur, particularly away from sections themselves.

To overcome the disadvantages of the loop method a slightly modified method to that used by Caro & van Tubergen (1962) was employed, as recommended by J. Pickett-Heaps (personal communication).

A perfectly clean photographic glass plate (emulsion removed) was placed in a flat tray (12 × 8 × 2 cm) and both warmed to approximately 70 °C.

A 3% solution of Difco agar was heated in an autoclave and poured over the glass plate to fill the tray and allowed to cool. The glass plate was removed from the tray and the surplus agar cut away. The agar slab was then cut into approximately 2.5 cm^2 blocks and a clean microscope slide pressed against each agar block. The microscope slides used for this purpose were modified by having a 5 mm glass edge glued perpendicular to one of the narrower edges such that when an agar block was supported on a slide, it butted up against the glass edge.

By inverting the combined glass plate, agar block and microscope slide and applying a gentle twisting action, the agar block could be made to break away from the glass plate leaving a smooth clean surface uppermost.

This surface was then given a short exposure (1 - 1½ minutes) under an infra-red lamp to dry surface moisture. The surface was then flooded with a 0.1% parlodion in amyl acetate solution and dried vertically.

In the darkroom, a film of emulsion in a loop, prepared as previously described, was lowered over the parlodion film on the agar block. By utilising this procedure one obtains a flat, evenly distributed monolayer of halide crystals.

The agar block was trimmed lightly around the edges and lowered into a clean water bath at an angle where the parlodion film and film of emulsion floated off. The modified raised edge of the supporting microscope slide prevented the agar block preferentially sliding into the water bath before the parlodion-emulsion film floated off. Provided the block was trimmed, little difficulty was experienced in separating the film in to the water surface. The emulsion film could be readily seen on the water bath surface in reflected light from the safelight.

Prepared grids were placed section down on the floating film, after which grids with attached film could be removed with a wire mesh.

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ENVIRONMENTAL EFFECTS ON EPICUTICULAR WAXES OF *BRASSICA NAPUS* L.

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Abstract

Under controlled environmental conditions, seedling plants of *Brassica napus* L. were subjected to three different temperature regimes and, by differential shading, to three light levels. Marked changes in wax ultrastructure were observed within the temperature range 15–27°C, varying from a pattern of single upright rodlets to one of flat, overlapping, dendritic platelets parallel to the leaf surface. The wax type was consistent at any particular temperature irrespective of light condition. The effect of reduction in light intensity level was an apparent reduction in surface deposition of wax. This was confirmed by gravimetric determinations of total yield of surface wax per unit area and per leaf.

I. INTRODUCTION

A number of studies on the ultrastructure of leaf surface waxes have pointed out the variation that can be expected between species (Mueller, Carr, and Loomis 1954; Schieferstein and Loomis 1956, 1959; Juniper and Bradley 1958; Juniper 1959*a*; Hallam and Chambers 1970) while others have indicated some degree of infraspecific variation (Hall and Donaldson 1963; Whitecross 1963; Hall *et al.* 1965; Hallam and Chambers 1970). Age effects on chemical composition of wax have also been noted (Martin and Batt 1958; Martin 1960) while the biosynthesis of *Brassica* waxes in particular has been studied in some detail (Purdy and Truter 1963; Kolattukudy 1965, 1966, 1968; Macey 1970; Macey and Barber 1970). Still other studies have shown or hinted at the influence of some environmental factors in determining the ultimate structure and composition of the cuticular covering (Skoss 1955; Juniper 1959*b*, 1960; Whitecross 1963; Hallam 1970).

The present study, under controlled environmental conditions, is concerned with changes in ultrastructure and quantity of surface wax deposition, as affected by temperature and light, on leaves of field rape, *Brassica napus* L.

II. MATERIALS AND METHODS

(*a*) *Growing Procedures and Conditions*

Seedlings of *B. napus* were raised in a naturally lit glasshouse of the CERES phytotron, CSIRO, Canberra, under a day/night temperature regime of 24/19°C. After establishment for 2 weeks at 21/16°, seedlings were transferred to their respective treatments as listed below.

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Temperature conditions consisted of three treatments: 27/22, 21/16, and 15/10°C. Light conditions consisted of three treatments: 100%, 60%, and 40% full glasshouse light. The 100% light treatment involved placing the pots on open trolleys in each temperature-controlled glasshouse. The reduced light treatments were achieved by growing plants in screened enclosures consisting of Sarlon plastic mesh arranged over metal frames standing on trolleys. Edge effects in all cases were avoided by growing two rows of non-experimental control plants around the perimeter of each trolley with experimental plants in the centre. Light intensities in the enclosures were checked against ambient conditions with a light meter, and all shade cloth used was checked and found to have even spectral qualities throughout the visible range.

All plants were given nutrient solution each morning and watered each evening. Those in the high temperature treatment (27/22°C) were given an additional midday watering to prevent any water stress which could have affected the results (Whitecross 1963).

(*b*) *Harvesting Procedure*

Sampling was carried out after plants had been in their respective treatments for 4 weeks. Disregarding the small leaves surrounding the growing point, the fourth visible leaf from the apex was taken as a representative sample from each plant. This leaf was fully expanded and mature, showing no signs of senescence. The main principle in this method of sampling was to compare leaves at a similar physiological age from the point of view of cuticular development. Sampling of a particular node number from the cotyledons was unsatisfactory for this particular study.

(*c*) *Electron Microscopy*

Shadowed carbon replicas of the adaxial surfaces of sample leaves were prepared by a modification of the method introduced by Juniper and Bradley (1958).

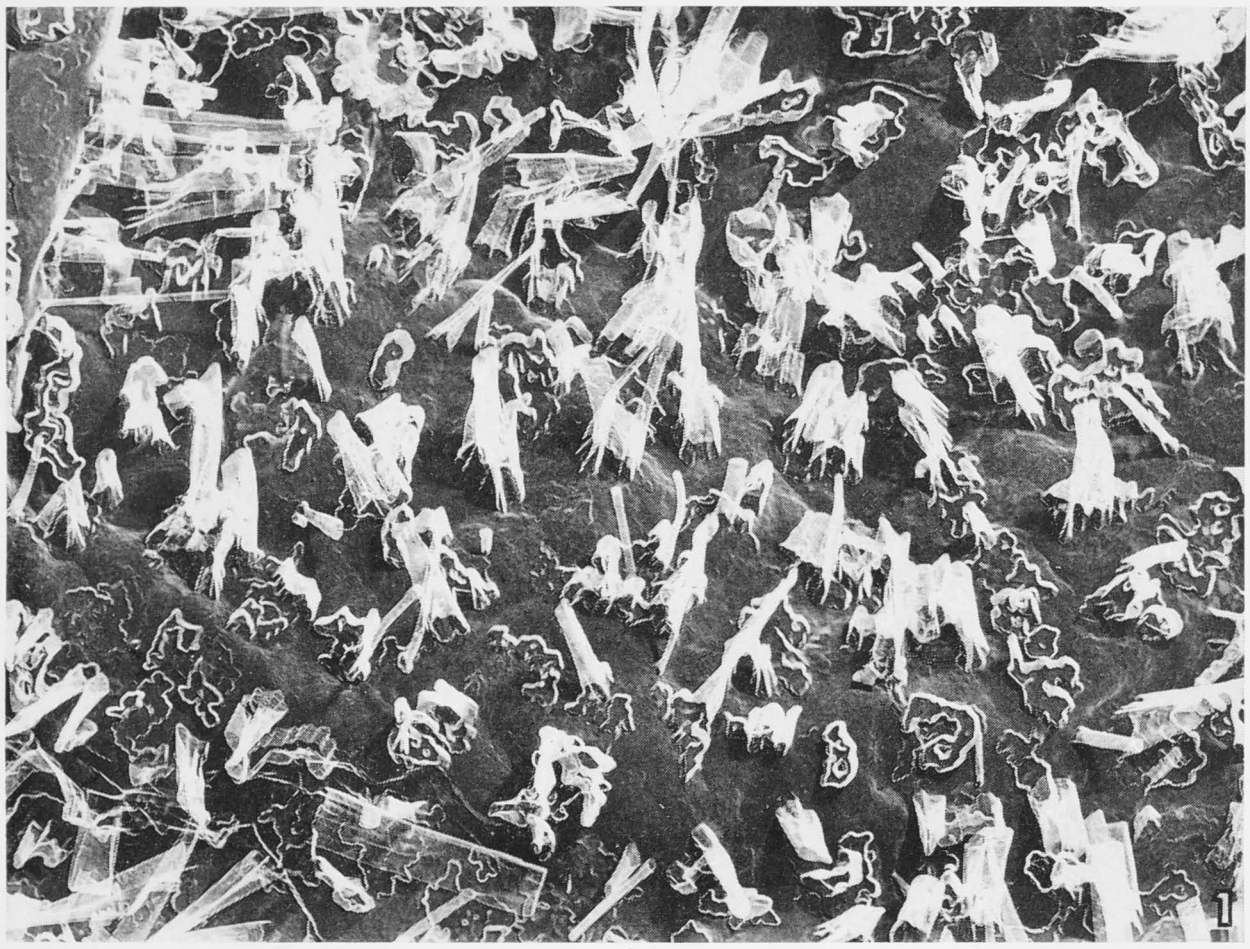
Small segments from each sample leaf were transferred to a microscope slide where they were fixed in place, adaxial surface uppermost, with Bedacryl 122X stock solution (40% in benzene). Being firmly stuck down in this manner prevented the segments from shrinking or becoming distorted during subsequent operations.

Preshadowing with platinum–carbon was carried out in the usual way, and the carbon replicas were stripped from the leaf surface with the aid of a single coating of Bedacryl backing film. Multiple backing layers (Juniper and Bradley 1958; Hallam and Chambers 1970) were found to be unnecessary for separating replicas from leaves of waxy species such as *B. napus*.

The combined plastic–carbon–shadow films were cut with a sharp blade into grid-sized pieces, transferred to uncoated grids, and the plastic removed with acetone. Grids were examined in a JEM-T6 electron microscope.

(*d*) *Determination of Wax Yield*

Undamaged leaves were sampled from several plants in each treatment. Leaves were dipped individually for 10 sec in each of four successive chloroform baths. This has been shown to remove all the surface wax effectively without causing internal

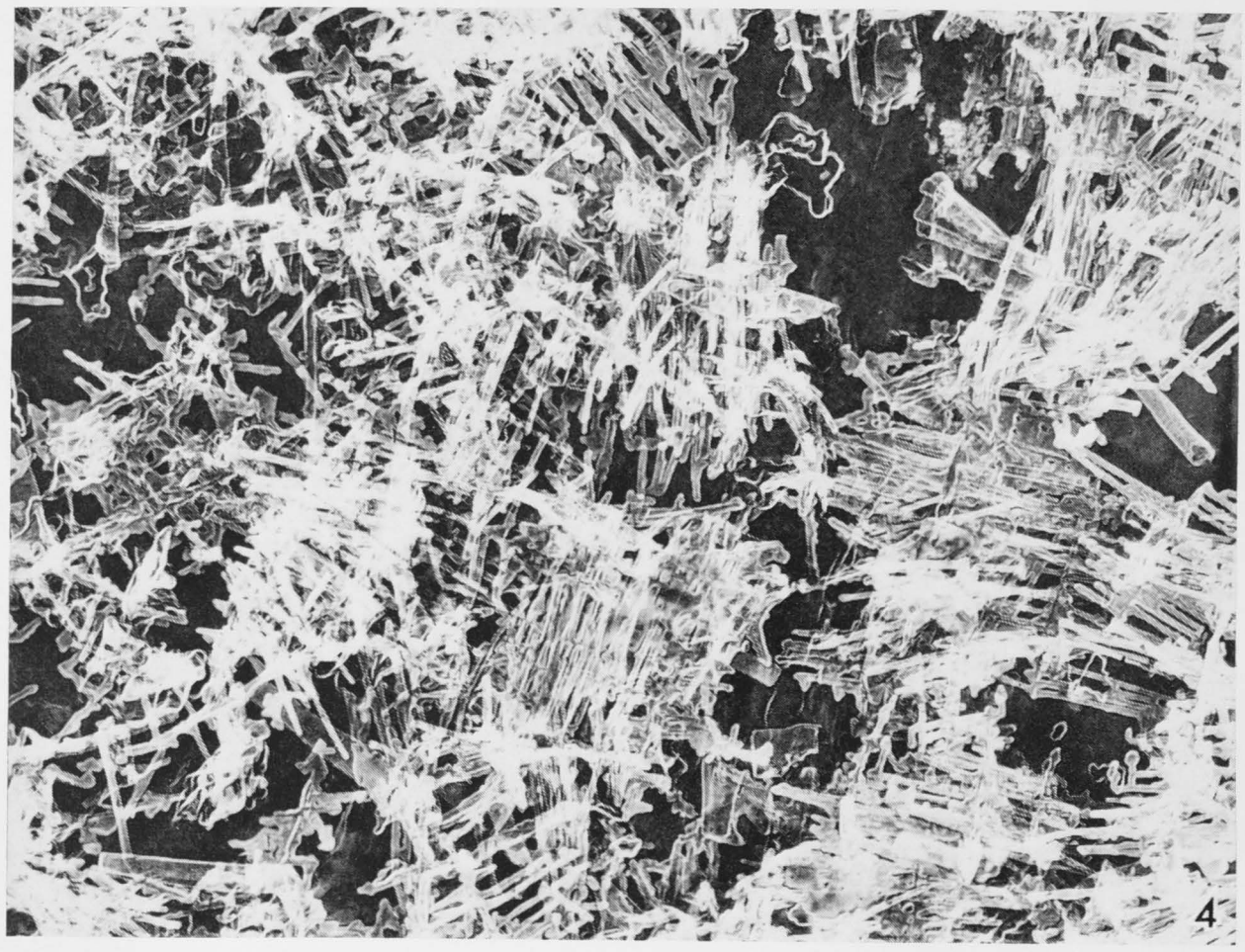


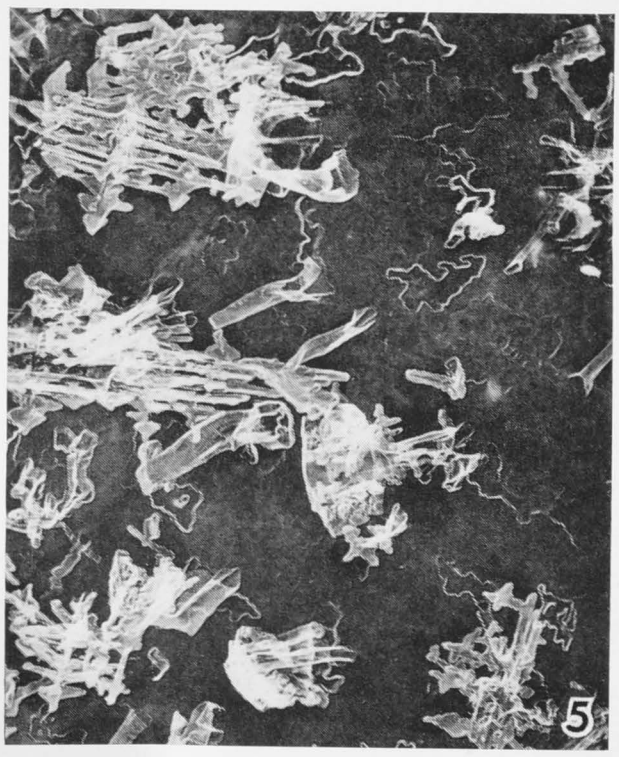
Figs. 1–3.—Electron micrographs of epicuticular wax on the adaxial surface of leaves of *B. napus* grown under different temperature and light regimes. Carbon replicas shadowed with platinum–carbon. × 4900.

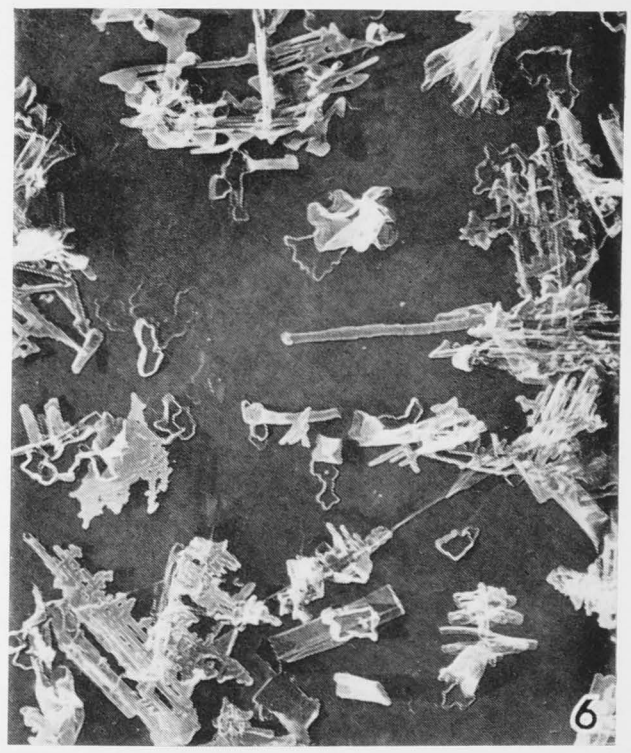
Fig. 1.—Grown at 15/10°C, full glasshouse light.

Fig. 2.—15/10°, 60% light.

Fig. 3.—15/10°, 40% light.







Figs. 4–6.—Electron micrographs of epicuticular wax on the adaxial surface of leaves of *B. napus* grown under different temperature and light regimes. Carbon replicas shadowed with platinum–carbon. × 4900.

Fig. 4.—Grown at 21/16°C, full glasshouse light.

Fig. 5.—21/16°, 60% light.

Fig. 6.—21/16°, 40% light.

disruption (Martin 1960). Following the last chloroform dipping, leaves were stored in 30% methanol for subsequent determination of leaf areas. The chloroform extract solutions were then bulked for each sample and the solvent removed in a rotary evaporator. Wax residues were then weighed.

III. Results

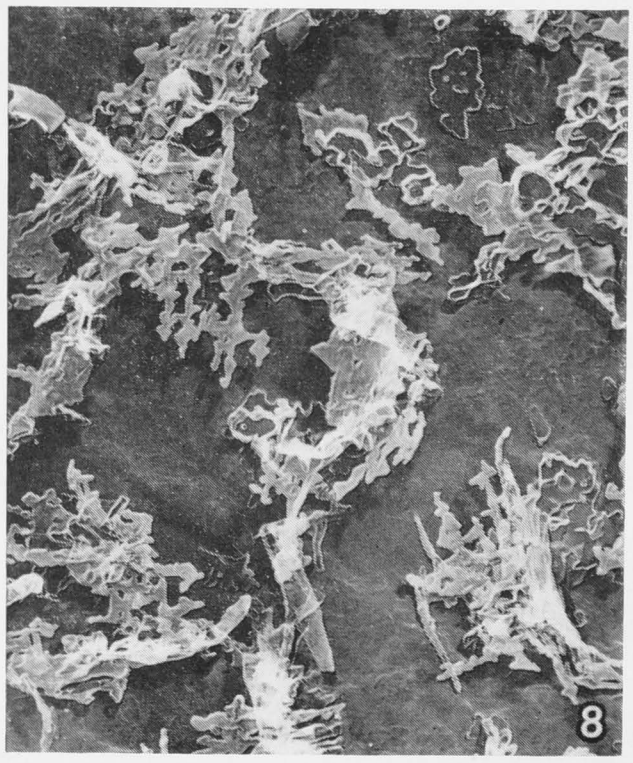
(*a*) *Ultrastructure*

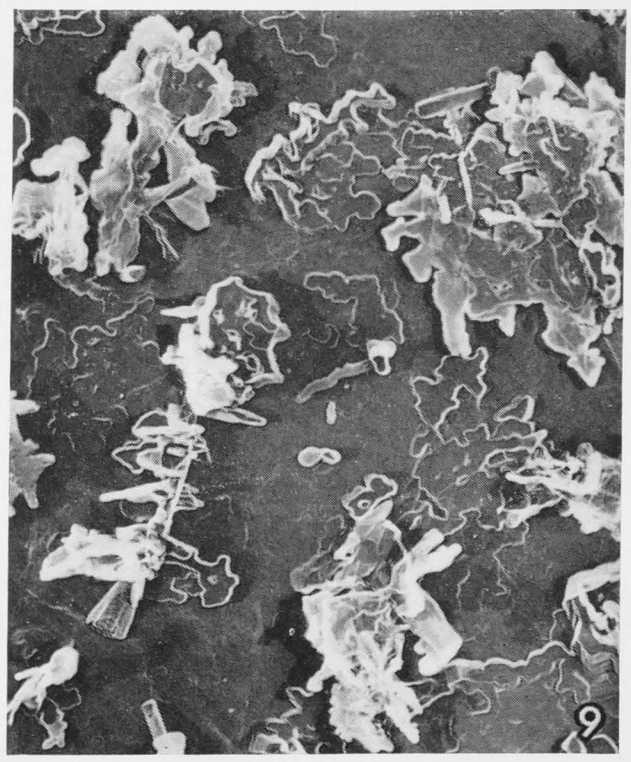
Leaf surface wax produced in the 15/10°C treatment consisted of rodlets of variable size but with a consistent characteristic "growth ring" pattern along their length (Figs. 1–3). In the full light treatment, rodlets measuring up to 3·4 μm were observed, but the mean length was closer to 1·8 μm. Base diameter of the rodlets ranged from 0·2 to 0·6 μm.

Reduction of light to 60% of full light resulted in a reduction in the dimensions of the wax rodlets (mean length 0·5–1·0 μm, with a maximum of 2·0–2·5 μm) and an even greater reduction in number of rodlets per unit area (Fig. 2). Further reduction of incident light, as in the 40% light treatment, led to the production of even smaller rodlets which rarely exceeded 0·7 μm in length. Incidence of rodlets per unit area remained approximately the same for the reduced light treatments.

Wax produced by leaves grown in the 21/16°C temperature treatment was morphologically different from that of the 15/10° leaves. The upright rodlets of the latter were replaced largely by flat platelets lying parallel to the cuticular surface. The platelets appeared to be formed from long axial strands having primary and secondary branches at right angles with some degree of fusion between adjacent branches, resulting in thin wafer-like structures (Figs. 4–6). In the full light treatment at this temperature (Fig. 4) the platelets appeared to be supported above the cuticular surface at a height of *c.* 0·3 μm, and they effectively covered more than 60% of the surface. As with the lower temperature, reduced light at 21/16° again caused a reduction in the dimensions and the complexity of individual crystallites, as well as in the surface coverage achieved. In 40% light in particular (Fig. 6) the wax structures were poorly developed though obviously of the same type. A sparse occurrence of the rodlet wax, characteristic of the lower temperature, could always be observed at all light levels.

At the highest temperature, 27/22°, the complex branching pattern of the waxes observed in the previous treatment was further extended. Fusion of primary and secondary branches of the platelets was more extensive than at 21/16°, so that platelets became practically solid sheets. In full light these platelets overlapped (Fig. 7) so that surface coverage commonly exceeded 70% of the total area. Smaller platelets and less frequent overlapping in the 60% light treatment gave the impression of a severe reduction in wax deposition (Fig. 8), while wax produced in the 40% light treatment was very irregular as well as smaller in size and lesser in quantity (Fig. 9). Rodlets of the low temperature type were still evident at this temperature, but they accounted for only a very small proportion of the total wax production.





Figs. 7–9.—Electron micrographs of epicuticular wax on the adaxial surface of leaves of *B. napus* grown under different temperature and light regimes. Carbon replicas shadowed with platinum–carbon. × 4900.

Fig. 7.—Grown at 27/22°C, full light.

Fig. 8.—27/22°, 60% light.

Fig. 9.—27/22°, 40% light.

(*b*) *Quantitative Wax Deposition*

Wax deposition, expressed as mean weight of wax per unit area or per unit leaf for each of the temperature and light treatments, is represented in Figures 10 and 11.

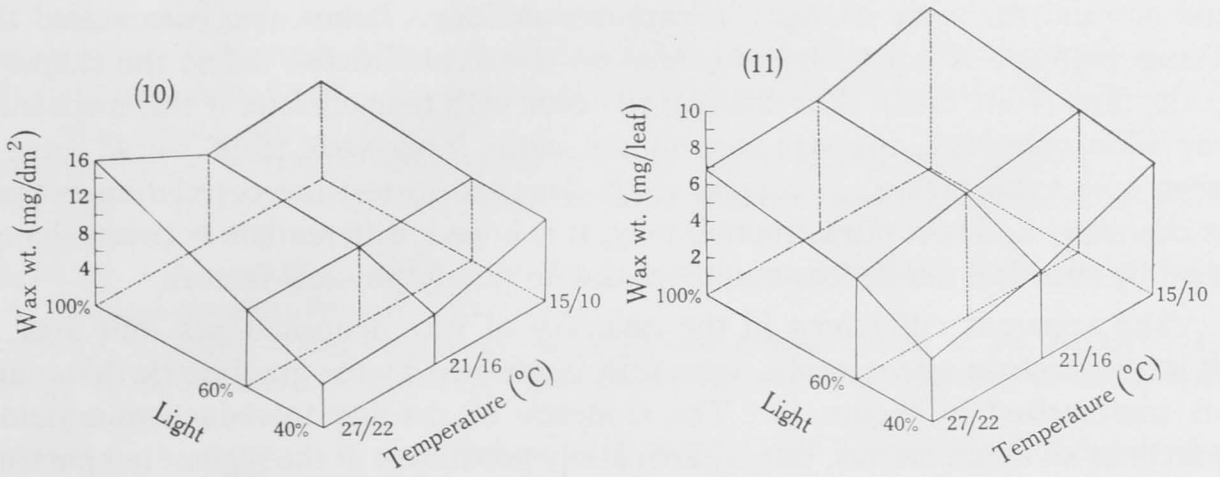


Fig. 10.—Mean wax yields per unit area of *B. napus* leaves grown under different temperature and light regimes (see Section II).

Fig. 11.—Mean wax yields per leaf of *B. napus* grown under different temperature and light regimes.

IV. Discussion

Electron microscopic examination of the surfaces of *Brassica napus* leaves grown under different environmental conditions revealed a qualitative response to temperature. It is clear that the ultrastructure of the wax crystallites changed markedly between the 15/10° and the 21/16° regimes. The change between 21/16° and 27/22° was smaller, being more one of degree than of basic type. A similar pattern of change was observed by Whitecross (1963) with the closely related species *B. oleracea* (cauliflower, cv. Pale Leaf), the only difference being that the transition from large rodlets to branched platelets occurred at a higher temperature. No such qualitative change, however, was evident in the study on *Eucalyptus viminalis* ecotypes (Banks and Whitecross 1971). In that species the shape of rodlets varied little with temperature within an ecotype, though some changes in surface distribution were observed.

The most likely explanation for the change in ultrastructure with temperature would be a change in the chemistry of the waxes produced, resulting in a different crystal pattern. The work of Macey (1970) on *B. oleracea* waxes would tend to support this hypothesis. On the other hand, waxes derived from *Brassica* spp. have been shown to be multicomponent mixtures, so that sufficient changes in the proportions of the constituents must result from the temperature differences to bring about the observed changes in ultrastructure. With no predominating chemical type, however, the situation with *Brassica* spp. is very different from that pertaining to the eucalypts, where Hallam and Chambers (1970) found a good correlation between the occurrence of "tube waxes", as evidenced in electron micrographs, and a predominance of β-diketones in the infrared spectral analyses of the waxes. Furthermore it was shown in a preliminary study (Whitecross 1963) that at least some of the morphological changes in wax crystals can be paralleled by fast or slow rates of crystallization

of wax from a solution in a wax solvent. Though virtually nothing is known about the form in which wax is transported to the cuticular surface, one theory is that it is deposited from a solution by dispersal of a solvent (Schieferstein and Loomis 1959). It is conceivable then that differential rates of dispersal at low and high temperatures could account for some changes in wax morphology. It has also been stated that different patterns of pores, demonstrated on dewaxed cuticles, define the shapes of wax deposits (Hall 1967). For changes to occur with temperature, if this mechanism alone were operative, the structure of the cutin framework itself would have to change with temperature. A current study designed to test the correlation between wax chemistry and wax ultrastructure may, it is hoped, differentiate between changes caused by chemical differences and those due to purely physical factors.

The apparent differences in the quantity of wax deposited per unit area, as seen in the electron micrographs, were fully confirmed by the gravimetric determinations summarized in Figure 10. The tendency of shading to reduce wax yield is apparent at all temperatures, but is particularly prominent at the highest temperature, 27/22°C. There is also a tendency for more wax to be produced the lower the temperature, which was also a feature of the temperature response of *Eucalyptus viminalis* (Banks and Whitecross 1971). The one high point at 27/22°, 100% light which is not in agreement with this trend might be due to a genuine increase in wax deposition.

With fluctuations in leaf area complicating the picture, it was speculated that the temperature and light treatments might not influence the amount of wax produced per leaf so much as the total area on which it is deposited. However, wax yield expressed on a per leaf basis and plotted against treatments (Fig. 11) showed an interaction similar to that evident in Figure 10. If anything the relationship is more clearly represented in Figure 11, with most wax per leaf produced by low temperature, high light conditions and the least by high temperature, low light.

V. Acknowledgments

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